ORIGINAL ARTICLE

Genetic factors associated with population size may increase extinction risks and decrease colonization potential in a keystone tropical pine

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Abstract

Pioneer species are essential for forest regeneration and ecosystem resilience. *Pinus chiapensis* is an endangered pioneer key species for tropical montane cloud forest regeneration in Mesoamerica. Human activities have severely reduced some *P. chiapensis* populations, which exhibited a small or null colonization potential suggesting the involvement of genetic factors associated with small populations. We explored the relationships between (i) population genetic diversity (allozymes) and population size, including sampling size effects, (ii) fitness estimates associated with colonization potential (seed viability and seedling performance) in a common environment and population size, and (iii) fitness estimates and observed heterozygosity in populations with sizes spanning five orders of magnitude. All the estimates of genetic diversity and fitness increased significantly with population size. Low fitness was detected in progenies of small populations of disturbed and undisturbed habitats. Progenies with the lowest observed heterozygosity displayed the lowest fitness estimates, which, in turn, increased with heterozygosity, but seed viability peaked at intermediate heterozygosity values suggesting inbreeding and outbreeding depression. Inbreeding depression appears to be the most immediate genetic factor in population decline. Conservation efforts should try to maintain large and genetically diverse populations, enhance gene flow by restoring connectivity between adjacent populations, and avoid genetically distant individuals.

Introduction

Modern ecosystem management strategies should include actions that enhance the resilience of ecosystems to environmental change. Such actions include improving the capacity of the ecosystems to return to desired conditions after disturbance, facilitating the transition of ecosystems from current to new conditions, and lessening impacts on climate (Millar et al. 2007). Some native pioneer (early successional) tree species play an important role in ecosystem dynamics by colonizing degraded areas, facilitating the subsequent establishment of shade-tolerant species by habitat modification, and reversing some of the negative effects of environmental degradation. Anthropogenic habitat destruction has drastically reduced the areas of native ecosystems and the size of many plant populations, particularly in the tropics, where deforestation has reached unprecedented rates in recent decades (Dirzo and Garcia 1992; Cayuela et al. 2006). Implementing forest management practices that use secondary tree species is an option that needs to be developed, particularly if such species are economically valuable.

Populations of some key species for forest regeneration in tropical areas have been critically diminished. Under such circumstances, individuals of these populations may exhibit a reduced performance jeopardizing even more

the persistence of such ecosystems. In general, low germination and low plant vigor are common in plants from fragmented tropical areas (Martínez-Garza and González-Montagut 1999; Cascante et al. 2002), and low seed germination has been identified as a major barrier for regeneration in highly fragmented tropical montane cloud forests of Costa Rica (Holl et al. 2000). One possible explanation for this poor performance is the involvement of genetic factors associated with small population size. This possibility needs to be explored particularly in tropical areas. Had the involvement of genetic factors be demonstrated, several actions can be undertaken to minimize such effects. These include implementing seed banks, clone banks, progeny tests, and seed orchards for more effective conservation of genetic diversity (Ledig 1988) and undertaking management and silvicultural practices that look at the genetic constitution of the regenerating stands (Ratnam and Boyle 2000).

Small population size is considered an important factor increasing the risk of extinction, owing to demographic and genetic stochasticity. Population genetic theory predicts increases in homozygosity and decreases in population genetic variability in small populations. These processes are expected to jeopardize population persistence by reducing population fitness through inbreeding depression and by decreasing the population's ability to respond to environmental changes (see reviews in Willi et al. 2006; Allendorf and Luikart 2007). Theoretical studies (Mills and Smouse 1994; Vucetich and Waite 1999) and experimental approaches in nonthreatened, short-lived plant species in temperate regions (Newman and Pilson 1997) have provided evidence of the role of genetic factors on population extinction. To implement effective conservation, restoration, and management practices in the tropics, there is a need to examine this role for long-lived tropical tree species, many of which are endangered or threatened. Nevertheless, these species have been neglected by previous research. Predicting the population genetic consequences of habitat deterioration in the tropics cannot easily be achieved (Alvarez-Buylla and Garay 1994). Habitat fragmentation has facilitated gene flow in some species, enhancing the opportunities of increasing population genetic diversity (White and Boshier 2000; Dick 2001), and decreasing genetic diversity in others (Lowe et al. 2005). However, a recent meta-analysis showed that trees, in particular tropical trees, are underrepresented in genetic studies of habitat fragmentation (Aguilar et al. 2008).

The genetic impacts on fitness associated with population size in long-lived species can be explored by assessing the relationships between (i) fitness and population size; (ii) population genetic parameters expected to be related to fitness or population persistence and population size;

and (ii) fitness, or a fitness component, and a population genetic property presumably related to fitness, such as observed heterozygosity. A few studies have explored these three relationships simultaneously even in temperate habitats (Ouborg 1993; Oostermeijer et al. 1994), and to our knowledge, this kind of study has not been performed for pioneer tropical long-lived plants.

At least two potential sources of bias should be considered when exploring the relationships between plant population size, fitness, and genetic variation. First, fitness may depend on both habitat quality and genetic factors. Small populations are usually associated with highly degraded habitats relative to those of larger populations. Therefore, when comparing the performance of plants from large and small populations, it is usually difficult to disentangle the effects associated with population size from those of habitat quality, which has been a common problem in previous studies (Leimu et al. 2006). This problem can be circumvented in two ways: (i) including in the analyses small populations from both conserved and degraded habitats, and (ii) estimating fitness in conditions where environmental effects are controlled, such as a common environment. The latter procedure allows exploring whether fitness changes have a genetic component. Second, most population genetic parameters are sensitive to sample size. As both genetic (effect of population size) and statistical (sample size) sampling may affect the results obtained (see Weir 1996), sample size effects need to be taken into account in the analyses.

Studies of the fitness effect of inbreeding through correlations of heterozygosity of molecular markers and fitness estimates are not exempt from methodological and interpretational caveats (Pemberton 2004; Hansson and Westerberg 2008). However, they are an option for species in which pedigree studies are unavailable or difficult to achieve, as is the case for most threatened and long-lived tree species. If inbreeding and drift are important constituents of current population fitness, then heterozygosity and population size should be positively correlated with fitness among populations of a species (Reed and Frankham 2003). The cost of inbreeding in natural populations has been recently reviewed (Crnokrak and Roff 1998; Keller and Waller 2002; Leimu et al. 2006). For threatened species of trees, this cost has been little investigated, despite the urgent need for such studies, given the potential role of genetic effects in population extinction (Leimu et al. 2006; Willi et al. 2006; Grueber et al. 2008; Hansson and Westerberg 2008). Trees might not display similar responses to those observed in other life forms with respect to inbreeding and population size. Outcrossing and gene flow are usually higher in trees, as is the mutational load, owing to a greater

individual longevity and an expected higher number of mitotic events per generation (Petit and Hampe 2006).

Pinus chiapensis (Mart.) Andresen (*P. strobus* var. *chiapen*sis Mart., Pinacaee) is an endangered tropical pine from mid-elevation (265–2200 m) humid regions in the mountains of southern Mexico and western Guatemala. This pioneer species colonizes degraded areas, such as those resulting from the abandonment of maize fields managed under the slash-and-burn system of cultivation. The forests dominated by *P. chiapensis* facilitate the establishment of shade-tolerant species typical of tropical montane cloud forest, the original vegetation (del Castillo and Blanco-Macías 2007; del Castillo et al. 2009). Therefore, *P. chiapensis* appears to be essential for the resilience and stability of this kind of ecosystem. Furthermore, this species is a valuable timber tree (del Castillo and Acosta 2002).

The capacity for invading open areas, a critical factor for survival in fragmented habitats (Primack and Miao 1992), varies widely among *P. chiapensis* populations and is probably associated with population size. Small populations have been severely reduced in recent decades, display very limited or absent regeneration even in adjacent open areas, and are under immediate threat of extinction (Zamora and Velasco 1977; Dvorak and Brouard 1987; Martínez-Carrasco 1998). By contrast, in some populations, forest fragmentation has opened new areas for colonization favoring population expansion. Regeneration in these large populations is abundant in open areas, even in the presence of human activities (Table 1). These differences in establishment among large and small populations suggest the involvement of genetic effects associated with population size, in which case such differences should persist in a common environment and be associated with the size of the source population. Clearly, there is a need to evaluate the genetic consequences of small population size to formulate strategies for effective conservation and sustainable management of genetic resources for species inhabiting degraded landscapes (Newton et al. 1999). If ignored, genetic effects associated with population size in *P. chiapensis* could hamper restoration efforts and jeopardize the resilience of tropical montane ecosystems subjected to disturbances, such as slash-and-burn agriculture.

This study documents the relationships between population size, genetic variation, and fitness estimators in *P. chiapensis*, taking advantage of the wide range of population sizes found in this species, spanning five orders of magnitude. To disentangle the effects of habitat quality

Table 1. Details of the populations of *Pinus chiapensis* used in this study, including population name, state (Mexico) or department (Guatemala), geographic coordinates, altitude, approximate population size, mean diameter at breast height (DBH), density of seedlings, and the number of sampled maternal trees.

Population	State/ Department	Altitude (m)	Latitude (N)	Longitude (W)	Mean DBH (cm)*	Approximate population size	Density of seedlings' 1000 (ha^{-1})†	Number of sampled trees
1. Xochitlán‡	Puebla	1095	19°58′	96°53′	56.6	2×10^2	0.00	18
2. Atzalan	Veracruz	780	19°22′	97°34′	53.6	10^3	0.00	21
3. Chiquihuitlán‡	Oaxaca	1162	18°00′	96°46′	48.0	10^3	0.05	19
4. Usila‡	Oaxaca	1430	17°47′	96°32′	70.8	20	0.00	7
5. Yerba Santa	Guerrero	1865	17°31′	99°58′	46.3	10^4	0.04§	16
6. El Rincón‡	Oaxaca	1737	17°21′	96°18′	28.1	10^5	17.2	158
7. Chayuco	Oaxaca	1770	17°13′	97°59′	56.9	2×10^2	17.8	6
8. San Juan Copala‡	Oaxaca	1555	17°11′	97°59′	38.0	1	0.00	1
9. Rio Pinal‡	Oaxaca	700	16°59′	94°36′	85.4	36	0.00	10
10. Chenalhó	Chiapas	1555	16°58′	92°44′	35.9	10^3	0.00	25
11. Tentic‡	Chiapas	1745	16°52′	93°09′	30.1	2×10^2	0.00	12
12. Escolapa	Oaxaca	265	16°51′	94°43′	74.9	2	0.00	2
13. Guevea	Oaxaca	815	16°48′	95°23′	47.8	2×10^2	0.00	15
14. Coatlán	Oaxaca	1365	16°10′	96°53′	50.6	103	0.00¶	17
15. Barillas‡	Huehuetenango**	1683	15°47′	91°19′	41.6	104	0.00	16

*Based on trees measured on the entire population (populations 5, 9, 14, 15), selected fragments (populations 8, 12), selected trees (populations 1, 2, 10), or plots usually of 0.1 ha (populations 3, 4, 6, 7, 11, 13).

†Seedling establishment determined based on a minimum of 50 m² of surface sampled. Seedlings were defined as plants ≤20 cm height and ≤2 years old.

‡Populations used in plant performance evaluations.

§Regeneration observed in open adjacent spots.

¶Owing to very low seed viability in this population, only seeds from three maternal plants could be analyzed electrophoretically.

**Guatemala.

from those of population size, we included small populations from both disturbed and conserved habitats and conducted our fitness estimations in a common environment. We also took into account sample size effects in the analyses.

Materials and methods

Study organism

Pinus chiapensis is a tall (∼50 m) neotropical white pine classified as Vulnerable by the IUCN Red List Criteria (Farjon and Page 1999). This pine is an important resource for epiphytic plants, animals, and humans, and a key species for both natural regeneration and restoration of tropical montane cloud forests in Mexico and Guatemala (Cordova and del Castillo 2001; González-Espinosa et al. 2008; del Castillo et al. 2009). Forests dominated by *P. chiapensis* are the first to appear after disturbance and play important environment services, including accumulation of carbon, shading the soil surface, fostering soil development, and facilitating the release of nutrients from the bedrock to the soil by increasing soil acidity (del Castillo et al. 2009).

The habitat of *P. chiapensis* is discontinuous owing to both topographic and anthropogenic factors. This species is restricted to the windward side of tropical humid mountain areas, usually above the tropical rain forest and below frost-exposed areas in disturbed places (see Newton et al. 2002; del Castillo et al. 2009). A study conducted on three populations revealed that the breeding system varies between full outcrossing in two populations to a mixed mating system with approximately 51% selfing in the largest population, suggesting that small population sizes enhance outcrossing in this wind-pollinated species (del Castillo and Trujillo 2008). Dispersal and gene flow in *P. chiapensis* are probably limited because of the following factors: (i) preliminary surveys on seed dispersal revealed that most of the seeds produced in the fragments (>95%) are dispersed no longer than 1.5 km from the border edge (R. F. del Castillo, unpublished results). (ii) Isozyme analyses conducted at local scale show evidence of genetic structure including subpopulation differentiation in Sierra Madre de Oaxaca, Mexico (Ramírez Toro 2005); and (iii) studies based on RAPDs (Newton et al. 2002) and quantitative traits (Dvorak et al. 1996), reveal a significant genetic differentiation among populations at regional scale.

At least three key factors apparently threaten the populations: (i) the continuous reduction, isolation, and degradation of many populations by land use change and timber overexploitation; (ii) an unusual low genetic diversity compared with other species of pine; and (iii) substantial inbreeding depression detected at the seed germination stage (Newton et al. 2002; Ramírez Toro 2005; Syring et al. 2007; del Castillo and Trujillo 2008).

Study populations and plant material

We sampled 15 populations of contrasting size throughout the range of *P. chiapensis* (Fig. 1, Table 1). A population was defined as a group of individuals of *P. chiapensis* occupying a uniform piece of land, clearly separated from other populations by distances not shorter than 10 km, and habitats distinct from those occupied by the population. Extensive walks, vehicle ridings, and the inspection of 1:70000 aerial photographs and satellite imagery, when available, helped to discern populations. Population size was defined as the number of trees in each locality with ≥10 cm diameter at breast height, which is roughly the number of breeding individuals. Population size was estimated in two ways. In the four smallest populations studied, all adult individuals were tallied. For the other populations, estimations were based on field surveys assessing the number of reproductive individuals in 0.1 ha sampling plots, and extrapolating the mean of such numbers to the total area of the forest fragment, which, in turn, was estimated directly in the field or by using remote sensing images.

The smallest populations were forest remnants in a matrix of agricultural fields and pasturelands. The exception was the Río Pinal population (Chimalapas), located in a widespread area of a well-preserved tropical rain forest. Adult mortality was unusually high, probably because of aging and self-thinning; in this population, 54.5% of the standing trunks were dead with a rotten stem core, and the average age of the trees estimated from growth rings was 93 year. The rest of the populations were probably younger, judging by the tree sizes (DBH, Table 1) and some age estimates, and consisted of fragments of secondary forest that have established on previous maize fields in tropical montane cloud forest or tropical rain forest (Escolapa) areas. None of them had evidence of massive adult mortality. Most forests in tropical areas in Mexico remained well preserved before 1877 (Challenger 1998). Taking such information and assuming a generation time of 20 years, which is approximately the lowest age for reproduction in *P. chiapensis* (R. F. del Castillo, unpublished observations), it is unlikely that reductions in population size related to human activities exceeded six generations [(2000–1877)/20] for most of the studied populations. For populations in Sierra Madre de Oaxaca (Chiquihuitlán, El Rincón, Guevea, and Usila populations), this may be an overestimate as large-scale forest exploitation started in 1948 (Vidal García Pérez 1998).

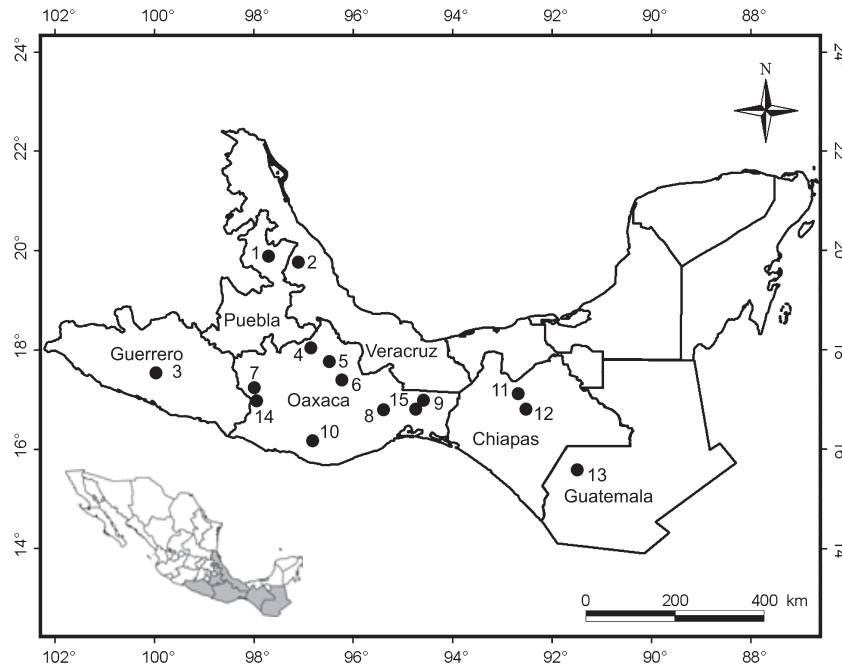In each locality, cones were collected by climbing trees separated usually at least 10–20 m from each other.

Figure 1 Map of Mexico and Guatemala showing the location of the *Pinus chiapensis* populations investigated in this study. Population numbers as in Table 1.

Voucher specimens were deposited at the herbarium of the Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional of Instituto Politécnico Nacional, Mexico (CIIDIR Oaxaca, OAX).

Allozyme analyses

Analysis of allozyme variation was conducted on seed radicles ground in 0.2 M phosphate buffer using 12% starch gel electrophoresis. Table 2 shows the enzymes analyzed, buffer systems, and staining protocols. A single seed

per maternal tree was used in the analyses. Limited number of viable seeds precluded the survey all the enzyme systems in some populations. We used a variable number of seeds and maternal plants reflecting between-population variation in number of maternal trees available for sampling and seed viability.

Fitness estimates

Seed germinability and seedling performance (growth and survival) were used as fitness estimators. Samples of 100

Table 2. Enzymes analyzed, enzyme commission number, buffer system, and staining protocols employed in the electrophoretic analyses.

Enzyme	E.C. number	buffer system	Staining method
Acid phosphatase (ACP)	3.1.3.2	Histidine-citrate, pH 7.0	Cheliak and Pitel (1984)
Alcohol dehydrogenase (ADH)	1.1.1.1	Histidine-citrate, pH 7.0	Levy (1989)
Aldolase (ALD)	4.1.2.13	Histidine-citrate, pH 7.0	Levy (1989)
Aspartate amino transferase (AAT)	2.6.1.1	Tris-citrate/LiB, pH 8.3	Levy (1989)
Cytosol aminopeptidase (CAP)	3.4.11.1	Tris-citrate/LiB, pH 8.3	Conkle et al. (1982)
Glucose 6-phosphate isomerase (GPI)	5.3.1.9	Tris-citrate/LiB, pH 8.3	Conkle et al. (1982)
Glutamate dehydrogenase (GDH)	1.4.1.3	Tris-citrate/LiB, pH 8.3	Conkle et al. (1982)
Isocitrate dehydrogenase (IDH)	1.1.1.42	Histidine-citrate, pH 7.0	Cheliak and Pitel (1984)
Malate dehydrogenase (MDH)	1.1.1.37	Histidine-citrate, pH 7.0	Levy (1989)
Menadione reductase (MNR)	1.6.99.2	Tris-citrate/LiB, pH 8.3	Conkle et al. (1982)
Nicotinamide adenine dinucleotide dehydrogenase (NADH+H)	1.6.99.3	Histidine-citrate, pH 7.0	Cheliak and Pitel (1984)
Phosphoglucomutase (PGM)	5.4.2.2	Histidine-citrate, pH 7.0	Cheliak and Pitel (1984)
6-Phosphogluconate dehydrogenase (PGDH)	1.1.1.44	Histidina-citrate, pH 7.0	Conkle et al. (1982)

seeds, less than 1 year old, per maternal tree were germinated on moist filter paper on Petri dishes arranged in a complete randomized design in the benches of a greenhouse at 26°C average temperature, which is close to the average temperature reported for natural populations of this species (Musálem and Téllez Pérez 2004). Seed germinability was defined as the fraction of seeds with emergent radicle. Ungerminated seeds were judged to be unviable because of their lack of enzymatic activity and their usually necrotic embryo. Therefore, seed germinability obtained in this way can be taken as the upper limit to the probability of seed germination and an estimate of seed viability.

Seedling survival and growth were used to evaluate seedling performance. A common garden experiment was conducted in the same greenhouse used for seed evaluations, by sowing seeds in plastic bags filled with soil from El Rincón locality, sieved in a 2-mm mesh. Most seeds did not germinate or the resulting seedlings died before the cotyledons protruded. A total of 520 live well-developed seedlings, that is, with protruded cotyledons, from 39 progenies and eight populations survived to conduct the experiment, which followed a complete randomized design. In each bag, a single seedling was left when more than one seedling emerged in the bags by carefully removing the extra seedlings. The first height measurement ($h1$) was taken 98.0 ± 6.2 day (mean ± SE) after sowing when the seedlings were approximately 6.2 ± 0.3 cm in height. Approximately 5 months after the beginning of the experiment (148.7 ± 6.2 day), a final height measurement was taken ($h2$) to each of the surviving seedlings. To evaluate the seedlings, we introduce a plant performance index, $PI(s)$, which considers simultaneously survival ($s = 1$, if the plant survived after, or died, $s = 0$, before the conclusion of the experiment), and the relative growth rate in height (cm):

$$PI(s) = \begin{cases} 0, & s = 0 \\ \frac{Ln(h2) - Ln(h1)}{t}, & s = 1 \end{cases}$$

t is the time interval (days) between $h2$ and $h1$. In *P. chiapensis*, growth is an indicator of the future performance of the plant, as both survival and fecundity tend to increase with plant size (del Castillo 1996, and unpublished results).

Data analysis

For each population, we calculated the following attributes: fraction of polymorphic loci (>0% criterion), mean number of alleles per locus, average expected heterozygosity (genetic diversity), an allelic richness estimator, and observed heterozygosity. The first three attributes are standard statistics developed to measure genetic variability

in samples (Charlesworth and Charlesworth 2010). However, they do not control for sample size differences, which also affect the estimates (see Weir 1996). The allelic richness estimator standardizes the samples from populations based on different sample size to a common sample size, usually the smallest of all the samples, using the rarefaction method (El Mousadik and Petit 1996). Therefore, this estimator is good at comparing genetic variation between samples of different sizes, but sacrifices information from populations with large sampling size. Finally, the observed heterozygosity is expected to be related to fitness if inbreeding and drift influence population fitness. The FSTAT program was used for parameter estimations (Goudet 2001).

To account for sample size effects in the genetic diversity estimators, we included sample size as partial correlate in all analyses exploring the relationship between genetic diversity and population size. We performed a regression analysis with a stepwise selection procedure for each of the studied population genetics attributes (Draper and Smith 1981). This procedure adds, one at a time, to a model with no variables, the predictor variable that produces the highest F statistics, and is statistically significant. We chose a $P \leq 0.15$ threshold for a variable to enter in the model and $P \leq 0.05$ for a variable to stay in the model using the REG procedure of SAS (v. 9.13, SAS Institute Inc 1990). Prior to statistical analyses, we transformed population size into a $\log_{10}$ scale to normalize residual variation. Our population genetics estimations of the smallest populations studied (San Juan Copala, Escolapa and Río Pinal) might have a major influence on the analyses as they are located at the lowest end of the range of the studied population sizes. However, they were based on very small sample sizes owing to small population sizes and scarcity of viable seed (Table 1). To check the robustness of the conclusions reached, we conducted another set of stepwise regression analyses as described earlier, but excluding these three populations, and calculated the allelic richness estimations based on a sample of 8 genes, in which the three smallest populations could not be included, and on a sample of two genes to include data of all studied populations.

To explore the relationship between population size and fitness, we regressed seed germinability and the plant performance index, to population size. The fitness estimates data could not be normalized nor the variances homogenized by standard variable transformations. Therefore, prior to statistical analyses, the data were replaced by their ranks, because this transformation is less likely to be distorted by non-normality and unusual observations (Montgomery 1985). To explore the relationship between fitness estimates and heterozygosity, the mean of the germination data and the plant performance

indices classified by population origin and heterozygosity class (1, 0–0.10; 2, >0.10–0.2; 3, >0.2–0.3, and 4, >0.3) were used as data entries. We used the RSREG procedure of SAS to detect linear and quadratic effects.

Results

Relationship between population size and genetic variation

All estimates of genetic variability used, that is fraction of polymorphic loci, mean number of alleles per loci, mean allelic richness at 2 and 8 sampled genes, mean predicted heterozygosity, and mean observed heterozygosity displayed a similar behavior with respect to population size, and were highly correlated among themselves ($r \geq 0.77$, $P \leq 0.008$). All of these estimates were significantly and positively correlated with population size when sample size was included as a covariate (Table 3). This variable was not significant during variable selection of the stepwise regression procedure for any estimator of genetic variability. At the end of this procedure, population size remained significant for all studied estimators of genetic variability. The final fitted regression equations explained between 62% and 87% of the total variation about the mean. Allelic richness based on a sample of eight genes (in which the three populations with the smallest sample size were left out of the model) increased more rapidly

with population size than when based on a two gene sample (Table 4, Fig. 2). In general, the same trends were observed when the smallest populations were excluded from the analyses, but the final r^2 values from the stepwise elimination procedure were always higher than final r^2 including all populations for each of the response variables. Also, slightly larger estimates of the parameters relating population size to the population genetic variables analyzed were obtained in this subsample compared to the analyses including the entire data set. However, for all genetic parameters, the subsample estimates were located within one standard error interval of the estimates obtained from the entire sample (Table 4).

Relationship between population size and fitness estimates

Seeds from 367 maternal trees and 15 populations were analyzed for germinability. The average germinability per population increased significantly with population size ($r^2 = 0.37$, $P = 0.01$, Fig. 3A). Our data suggest that a decrease in one order of magnitude in population size is associated with 6.7% decrease in germinability on average. A total of 520 plants from eight populations were analyzed in a common garden experiment (Table 1). The plant performance index, which considers both survival and growth, increased significantly with the size of the

Table 3. Summary of the results of the correlation analyses (correlation coefficient and one tail significance probability in parentheses) relating allelic richness based on a random sample of 2 and 8 genes per population, the fraction of polymorphic loci, the mean number of alleles per locus, average expected heterozygosity (genetic diversity), and average observed heterozygosity per population with population size (log10 transformed). Sample size was included as correlate in the analyses of population size. See methods for details.

	Allelic richness (2 genes)	Allelic richness (8 genes)	Polymorphic loci	Alleles per loci	Expected heterozygosity	Observed heterozygosity
Population size	0.61 (0.031)	0.69 (0.014)	0.78 (0.004)	0.75 (0.006)	0.70 (0.011)	0.68 (0.015)

Table 4. Final results of the regression analyses using the stepwise elimination procedure for: allelic richness based on a random sample of 2 and 8 genes per population, the fraction of polymorphic loci, the mean number of alleles per locus, average expected heterozygosity (genetic diversity), and average observed heterozygosity related with population size (log$_{10}$ transformed, N). Equations include all populations studied (second column from left to right), or excluding the three populations with the smallest sample size (Escolapa, Río Pinal and San Juan Copala, fifth column).

| Response variable	Equations including all populations	r^2	P	Equations excluding the three populations with the smallest sampling size	r^2	P
Allelic richness (2 genes)	$y = 1.03 + 0.05\ N$	0.39	0.013	$y = 1.00 + 0.05\ N$	0.42	0.023
Allelic richness (8 genes)	–	–	–	$y = 0.93 + 0.19\ N$	0.36	0.040
Polymorphic loci	$y = 0.02 + 0.12\ N$	0.76	<0.001	$y = -0.08 + 0.14\ N$	0.71	<0.001
Alleles per loci	$y = 1.03 + 0.05\ N$	0.53	0.002	$y = 0.84 + 0.19\ N$	0.60	0.003
Expected heterozygosity	$y = 0.02 + 0.04\ N$	0.53	0.002	$y = -0.01 + 0.05\ N$	0.55	0.006
Observed heterozygosity	$y = 0.00 + 0.03\ N$	0.44	0.007	$y = -0.06 + 0.05\ N$	0.59	0.003
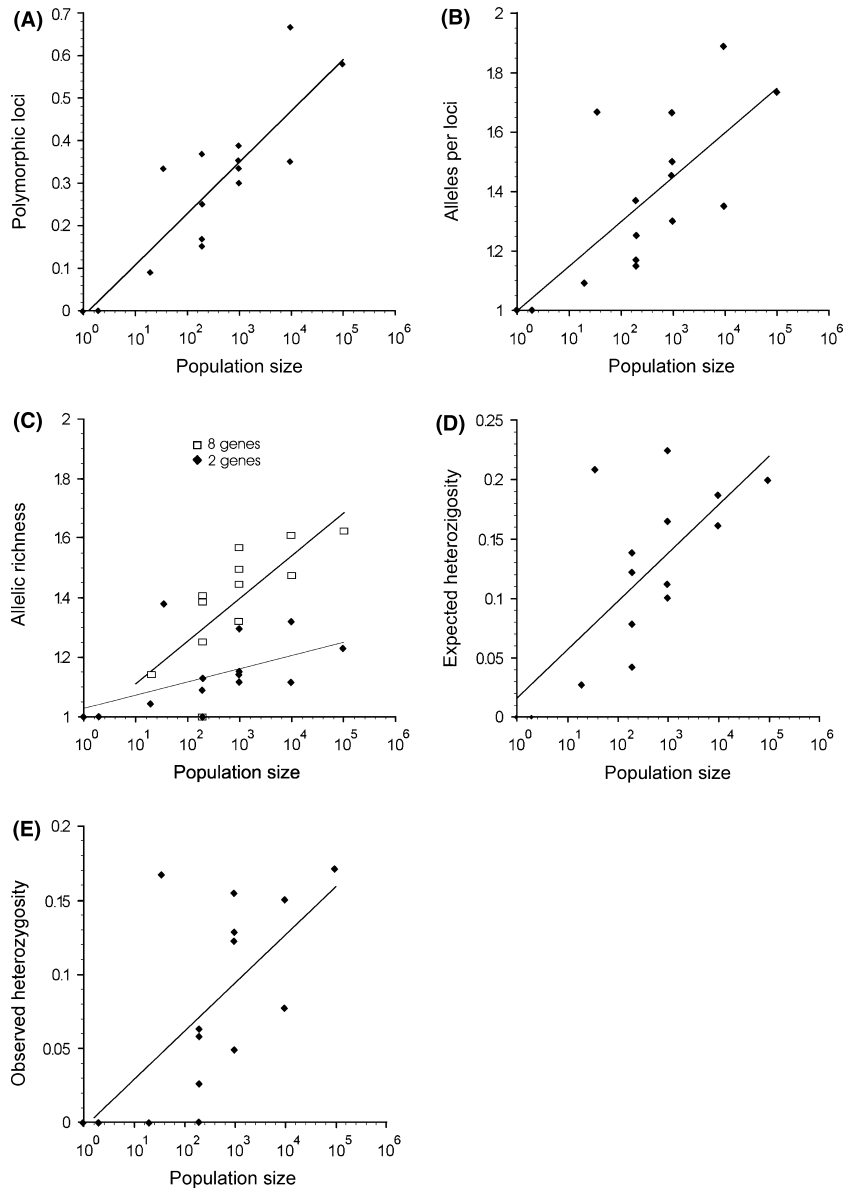
Figure 2 Positive associations between population size and (A) the fraction of polymorphic loci; (B) the mean number of alleles per locus (uncorrected for sample size); (C) the mean expected number of alleles (allelic richness) in a random sample of 2n and 8n genes per population; (D) expected heterozygosity; and (E) observed heterozygosity in *Pinus chiapensis*. The least square fitted lines are also shown. See methods for details.

population of origin ($r^2 = 0.74$, $P = 0.006$, Fig 3B). Thus, plants from larger populations probably will have higher probabilities of surviving the earlier stages of the life cycle than those of small populations other things being equal.

Relationship between fitness estimates and heterozygosity

At the population level, both germinability ($r^2 = 0.23$, $P = 0.03$) and the plant performance index ($r^2 = 0.21$, $P = 0.05$) were significantly associated with heterozygosity (Fig. 4). For both attributes, the lowest performance was observed at the lowest heterozygosity levels. For germinability, the linear ($P = 0.04$) and the quadratic ($P = 0.05$) component were significant, suggesting a curvilinear relationship with heterozygosity, with a peak at mean heterozygosity levels between 0.2 and 0.3.

Discussion

Population size and population genetic attributes

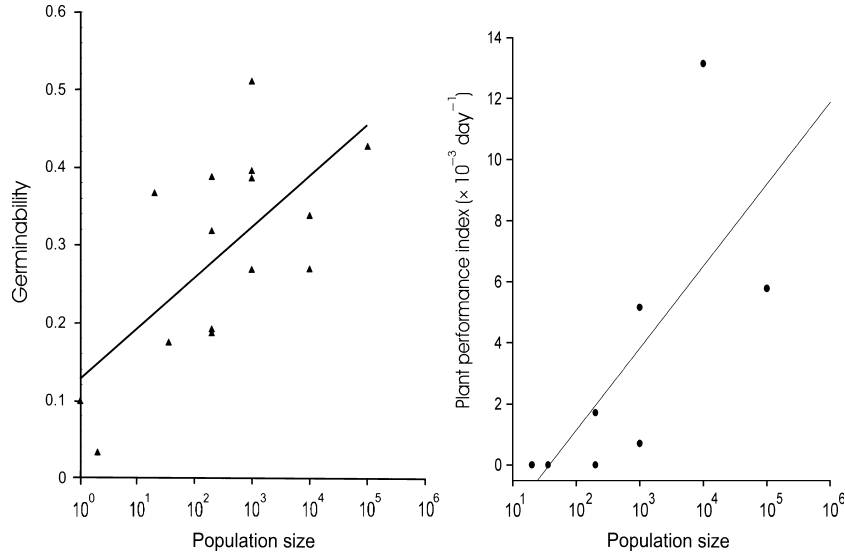Our results show a strong and consistent relationship between population size and genetic variation in

Figure 3 The relation of average population germinability and plant performance to population size. The least square fitted line is also shown.
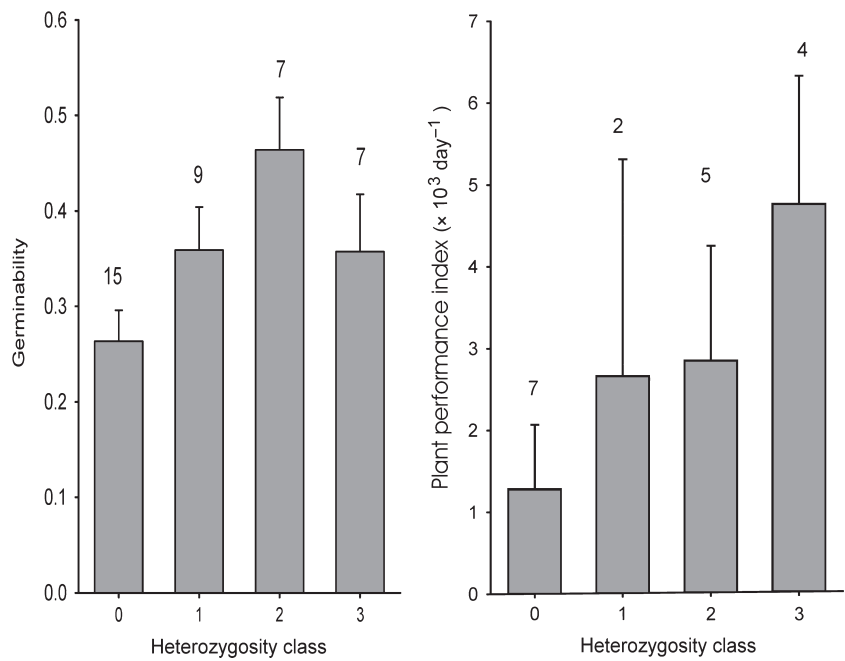

Figure 4 The relation between germinability (mean ± 1 standard error) and plant performance with mean heterozygosity classes (1, 0–0.10; 2, >0.10–0.2; 3, >0.2–0.3, and 4, >0.3). Sample sizes (number of populations) per class are shown above the error bars.

P. chiapensis. Sampling size did not affect the strength of the associations between population size and genetic variation, in agreement with Leimu et al. (2006) meta-analyses. All standard estimators of genetic variation used, including or excluding the three smallest populations, and the allelic richness estimators, which directly correct for sample size differences, increased in an approximately

linear fashion with the logarithm of population size, and explained a highly significant proportion of the total variance in their explored relationship with population size. The consistency of our results using different population genetic parameters points toward the robustness of our conclusion regarding the relationship between population size and genetic variation. Although these parameters tend

to be correlated between each other, studies conducted on other tree species show that they do not always correlate consistently with population size (e.g., Mathiasen et al. 2007). Furthermore, most studies on tropical plants trying to assess the effect of anthropogenic habitat degradation and fragmentation on parameters such as allelic diversity have found no significant consequences, although progeny inbreeding has resulted in significant impacts (Lowe et al. 2005). The latter result is consistent with ours, as the observed heterozygosity of progenies decrease toward small-sized populations.

Although most population genetic parameters behave relatively similar, it is instructive to pay attention to the Rio Pinal population. This population appears to have declined only recently, probably within one generation, judging by the high frequency of standing dead trees, a feature not observed in any other population. This population displayed one of the highest levels of allelic richness despite being one the smallest populations sampled, yet the levels of heterozygosity detected were nil. This result is in agreement with theoretical studies showing that during population decline, losses of genetic diversity take several generations to be detectable, whereas inbreeding increases immediately (e.g., Amos 1999; Lowe et al. 2005). The decline of genetic diversity owing to habitat degradation detected in the other studied populations is expected to be relatively rapid, considering that reductions in population size associated with anthropogenic habitat destruction probably took no more than six generations in the most extreme cases, as explained earlier. In some small populations, genetic variation was undetectable. A decline in allelic diversity during the first generations after a population bottleneck is expected to be faster with low-frequency alleles than with equally frequent alleles (Fuerst and Maruyama 1986) and may help explain the decline in allelic diversity observed in the small sized populations studied.

Population size and fitness estimators

Population mean seed germinability and plant performance decreased significantly with population size. All of the smallest studied populations had an extremely low germinability. In Río Pinal, only 25% of the sampled trees had viable seed. In Escolapa, only one of the two living trees produced viable seeds. Such low figures were not detected in any population larger than 50 adult individuals. The previous results and the lack of recruitment observed in the field suggest that short-term extinction is the most likely fate of such populations. Several studies performed mostly in annuals or short-lived perennials have demonstrated significant decreases in fitness components, including seed germination, in declining or small

populations (Menges 1991; Ellstrand and Elam 1993; Ouborg and Treuren 1994; Groom 1998). Our results show that similar reductions not only in germinability but in the survival and growth of young plants can be detected in progenies from small populations of a pioneer tropical tree with a long life span and living in subdivided habitat.

As explained previously, colonization is crucial for pioneer species, such as P. chiapensis. Once established, pioneer species modify the habitat in such way that further establishment is precluded. Newly created habitats by disturbance are an absolute requirement for a successful establishment in this kind of species. Depending on the productivity of the site and the predictability of the disturbance, plant species may develop different ecological strategies. In sites with high rainfall and unpredictable disturbances, such as those of P. chiapensis, rapid growth and tall stature are essential, before the sites are closed to invasion by more shade-tolerant trees (Keeley and Zedler 1998). Therefore, our fitness estimators could be taken as indicators of the colonization potential. In such case, our results provide evidence that the colonization potential of P. chiapensis is closely associated with population size. The fact that this tendency prevails in a common and benign environment suggests the involvement of genetic factors on the poor regeneration capability observed in small populations. Thus, the low or null recruitment observed in the field in small populations may have both genetic and ecological bases. The relative contribution of both factors remains to be explored. However, the very low viability of the seeds in these populations suggests that this attribute is a major constrain for recruitment.

Disturbance has been frequently associated with small population size. However, depending on its magnitude and frequency, habitat disturbance can have opposite effects on population size, and thereby on genetic diversity, in species such as P. chiapensis. A disturbance with frequency low enough to allow plant reproduction can create open habitats for colonization and boost population size, as is the case of El Rincon population (del Castillo and Blanco-Macías 2007). If severe or very frequent, disturbance can hamper successful plant establishment and eventual reproduction, as appears to be the case of Escolapa and San Juan Copala populations. Therefore, comparison of population genetic variation between degraded and conserved stands, as has usually been performed in studies of tropical plants, can lead to inconsistent results (see Lowe et al. 2005). This may help explain the lack of significant impacts detected in other species. In P. chiapensis, small population size, rather than disturbance, appears to be a major factor in population decline, as small populations of both conserved and disturbed habitats displayed low colonization potential.

Fitness traits related with colonization potential and heterozygosity

The genetic nature of the low fitness observed in small populations of *P. chiapensis* requires further research. However, this study provides evidence that inbreeding effects on fitness may contribute to explain the poor performance observed in small populations. Low heterozygosity was associated with low germinability and plant performance, suggesting the involvement of inbreeding depression. This result supports that of a previous study of inbreeding depression in *P. chiapensis* at family level based on three populations (del Castillo and Trujillo 2008). However, the present study with a larger heterozygosity range and at population level found that germinability decreased only at low heterozygosity levels. Above mean heterozygosity levels between 0.2 and 0.3, germinability tends to decrease. The observed heterozygosity of the 15 populations analyzed (0.047 ± 0.038, mean and SD) is much lower than the estimated threshold value above which increases in heterozygosity are expected to decrease germinability, indicating that in general inbreeding is associated with low germinability under natural conditions. The curvilinear nature of the relationship between heterozygosity and germinability could be the result of a combination of outbreeding and inbreeding depression (Schierup and Christiansen 1996), or maternal and nonmaternal effects of inbreeding acting on opposite directions as has been shown in a simulation study (del Castillo 1998).

Inbreeding depression is expected to be high in small populations, particularly during the first generations after a bottleneck (Kirkpatrick and Jarne 2000). Indeed, all of the smallest populations studied had very low or undetectable levels of heterozygosity coupled with very low levels of seed germinability and plant performance, which may explain the lack of recruitment in the field even in open places. Thus, inbreeding depression is likely to be a significant factor in population decline for small populations of *P. chiapensis* by decreasing the performance of the progeny. This decrease in turn may accelerate further population size reduction. This positive feedback suggests that inbreeding associated with small population size in *P. chiapensis* may be not only a consequence but a cause of population decline.

The persistence of inbreeding depression in *P. chiapensis* could be explained by two facts. First, inbred individuals hardly survive to reproduction, minimizing their seed contribution to the following generation (del Castillo and Trujillo 2008). Thus, the opportunities of purging are likely to be low. Second, intraspecific competition is likely to be strong, particularly at early stages of the life cycle (plant density declines approximately two orders of

magnitude from seedling stage to the stage of young prereproductive trees, owing to self-thinning), and to take place mostly between related individuals because dispersal is restricted and few individuals contribute to majority of the seeds in a given place (R. F. del Castillo, unpublished results). The previous conditions are expected to maintain deleterious alleles related with resource acquisition at high frequency, thus causing elevated load and inbreeding depression (super-soft selection, Agrawal 2010). Alternatively, inbreeding depression could be caused by other factors such as overdominance, in which case, inbreeding magnifies inbreeding depression. High levels of inbreeding depression are a common feature of pines from a broad variety of habitats (El-Kassaby et al. 1987; Kärkkäinen and Savolainen 1993; Sorensen 1999; Madriz Masis 2005; Politov et al. 2006; Bower and Aitken 2007). This study shows that inbreeding depression could be associated with population decline in *P. chiapensis*. Other genetic factors such as accumulation of mildly deleterious mutations (Lynch et al. 1995) or decreasing adaptability by reducing of genetic diversity (see Allendorf and Luikart 2007) cannot be ruled out as potential factors increasing extinction risks in *P. chiapensis*. However, given the rapid increase in inbreeding detected with decreasing population size and their relationship with viability, inbreeding depression appears to be the most significant genetic factor in population decline for small populations of *P. chiapensis* in the short term.

Fitness and genetic markers

What causes a significant correlation between isozyme heterozygosity and fitness in *P. chiapensis* is unknown. Direct selection on allozymes probably cannot explain this result, as they are likely to be effectively neutral particularly for small populations Selection coefficients at least equal to $1/2Ne$, where Ne is the effective population size, are required for an allele to be affected by selection (Kimura 1983). This requires a $Ne \geq 333$, considering that selection coefficients estimates for allozymes are usually low, 0.0015, or even less (Lynch 1987; Kreitman 1996). The Ne of most of the small studied populations of *P. chiapensis* is probably much smaller than this figure, because of their low population size (N), frequent bottlenecks during colonization, and their low or undetectable heterozygosity. For large populations with constant sizes, nearly neutral markers such as isozymes may correlate poorly with traits under selection (Reed and Frankham 2003). This may not occur in small populations. High levels of population linkage disequilibrium are expected to be generated by frequent bottlenecks in subdivided populations subject to extinction and recolonization (Ohta 1982). Furthermore, mixed mating systems,

detected in some *P. chiapensis* populations, are expected to generate identity disequilibrium (Hartl and Clark 1989; Vargas and del Castillo 2001).

Conservation and restoration implications

Genetic factors, in particular inbreeding depression, are key factors for the decreasing population viability in small populations of *P. chiapensis*. Given the key role of this species in forest regeneration in tropical montane cloud forest, any restoration effort and conservation action in this forest should consider the role of genetic factors in the population decline of this species. Restoring a small and degraded population necessarily requires using not only local seed sources but a high diversity and large numbers of seeds from other sources, in agreement with Broadhurst et al. (2008). This practice would augment the probabilities of successful establishment given the low germination rates detected in this species. Furthermore, sowing a genetically diverse pool of seeds and allowing the resulting seedlings to freely compete between each other would allow hardening the level of selection against deleterious alleles related with resource acquisition and decrease the mutational load (see Agrawal 2010). In the medium term, this would reduce the probabilities of inbreeding and inbreeding depression in the seeds produced in the resulting stands and would help to the maintenance of a larger population and therefore a larger genetic pool is needed for adapting to a changing environment. Thus, assisted migration is recommended not only for addressing climate change (McLachlan et al. 2007), but for maintaining a large gene pool. Our fitness inbreeding curve suggest that the risks of outbreeding depression at the level of seed viability cannot outweigh those of inbreeding depression within the levels of inbreeding detected in natural pollinated seeds. Nevertheless, avoiding seeds from very distant sources would be recommended as a cautionary measure to avoid the potential effects of the outbreeding depression in the resulting generation from which effects are unknown. Given the restricted seed dispersal detected in this species and in those of other trees of tropical montane cloud forest (del Castillo and Pérez-Ríos 2008), population size and proximity to both degraded areas and late successional forest would be key factors for maintaining a resilient landscape in these areas (del Castillo and Blanco-Macías 2007; Newton et al. 2009). Large and genetic diverse populations of *P. chiapensis* near disturbed areas such as abandoned corn fields would allow successful colonization and faster and successful revegetation. The proximity of such stands to late successional forest stands would allow a successful transition from secondary forest dominated by *P. chiapensis* to a mature tropical cloud

forest. Given the high impact of *P. chiapensis* in tropical montane cloud forest maintenance and regeneration, sustaining genetic diversity and heterozygosity in this species would have implications that go well beyond that of population level conservation. This study, therefore, provides evidence of the importance of genetic factors in the conservation and restoration of tropical areas.

Acknowledgements

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