

Effect of inbreeding depression on outcrossing rates among populations of a tropical pine

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Summary

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- Inbreeding depression is common among plants and may distort mating system estimates. Mating system studies traditionally ignore this effect, nonetheless an assessment of inbreeding depression that may have occurred before progeny evaluation could be necessary.
- In the neotropical *Pinus chiapensis* inbreeding depression was evaluated using regression analysis relating progeny *F*-values with seed germinability, the mating system was analysed in three populations with contrasting size, using isozymes, obtained a corrected outcrossing rate.
- Selfing decreased seed viability by 19%, relative to an outcrossed plant. Multilocus outcrossing rates, t_m , varied widely among populations. In the two smallest populations $t_m \cong 1$. Therefore, inbreeding depression did not affect the estimates, but overestimated t_m by 10% in the third population, which has a true mixed mating system (selfing was the major source of inbreeding), and an unusually low t_m for pines ($t_m = 0.54$, uncorrected, $t_m = 0.49$, corrected).
- Inbreeding depression may be an uneven source of bias for outcrossing estimates even at the infraspecific level. Accuracy but not precision may be gained by including inbreeding depression in outcrossing estimates. Therefore, caution should be taken when comparing t_m among species or even populations within the same species.

Key words: conifers, inbreeding depression, mating systems, outcrossing rates, *Pinus chiapensis*, population size.

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Introduction

Mating systems play a central role in determining population genetic structure (Hartl & Clark, 1989). In particular, they influence the levels and dynamics of genetic diversity, effective population size and population subdivision, all of which may affect population performance and persistence. Selfing, at one extreme of the mating system variation, favors population differentiation, local adaptation, and the expression of recessive alleles. At other extreme, the random union of gametes, random outcrossing, promotes gene flow, homogenizes populations, increases heterozygosity, and favors gametic linkage equilibrium. The use of marker alleles, such as isozymes, and the development of genetic models make possible precise, but not necessarily accurate, estimates of plant mating system parameters (see

Ritland, 2002). Mating system estimations are based on the proportion of heterozygotes carrying one allele that could not be of maternal origin. Therefore, such a proportion should not change by selection between the time of mating and progeny evaluation (Clegg, 1980). If inbreeding depression, δ , has already occurred at that time interval, heterozygosity should increase. Therefore, the estimated selfing rate (secondary selfing, s_m) may be biased downwards relative to the actual selfing rate ($s = 1 - t$, $t =$ outcrossing rate). This outcome may take place even with neutral marker loci if they are linked to a nonneutral locus. Furthermore, marker locus, such as isozymes, may not be completely neutral (Kreitman, 1996). Following Maki (1993), s can be estimated from s_m and δ :

$$s = s_m / (1 - \delta + s_m \delta), \quad \text{Eqn 1}$$

Since the difference between s and s_m increases with δ , comparisons of mating system estimates among species or populations may be inaccurate unless such estimates are corrected for δ . Nevertheless, such a correction has seldom been done (Husband & Schemske, 1996), and may be difficult to obtain for certain plant species. Thus, indirect approaches are required.

Mating systems depend on the particular attributes of the species' reproductive biology and spatial structure (Boshier, 2000). Thus, changes in population size and degree of isolation are expected to affect mating system. Such factors can be severely modified by deforestation and change of land use. Therefore, mating systems of many tropical tree species are likely to be altered, as tropical forests are being degraded and destroyed faster than any other biome (Myers, 1986). Relatively low rates of outcrossing are expected in small or isolated forest stands as pollen is derived primarily from a few sources, whereas outcrossing is expected to increase in dense and large stands (Mitton, 1992). However, this pattern may not be true for tropical species. A recent review, for instance, revealed that outcrossing is relatively common among the neotropical trees studied despite their typical low densities, but important and distinctive groups have been overlooked (Ward *et al.*, 2005). Indeed, most studies of mating systems in tropical ecosystems have focused on species of lowland tropical areas (Bawa *et al.*, 1985; Ward *et al.*, 2005). Very little is known about mating systems in plants from other kinds of tropical ecosystems.

Pinus chiapensis (= *Pinus strobus chiapensis*) is a species of particular interest in secondary tropical humid montane forest areas. Some populations are severely reduced, isolated and degraded owing to change of land use and overexploitation, since this pine is used for timber because trees can reach

> 45 m height and its wood is whitish and lightweight (Zamora & Velasco, 1977; del Castillo & Acosta, 2002). As a result, this species is classified as 'vulnerable' according to the International Union for the Conservation of Nature and Natural Resources (IUCN) Red List criteria (Farjon & Page, 1999), and as one that requires 'special protection' by the environmental agency of the Mexican Government (Secretaría del Medio Ambiente y Recursos Naturales, 2002). In few areas, however, populations are expanding over areas originally occupied by tropical montane cloud forest (del Castillo & Blanco Macías, 2007). Many seeds produced from trees in small and declining populations are unviable (R. F. del Castillo *et al.* unpublished). Inbreeding depression exacerbated by increases in consanguineous matings in these populations may account for such results, but studies on mating systems are absent. The goals of this study are: (1) to evaluate the mating system in three populations of *P. chiapensis* with contrasting size; (2) to estimate inbreeding depression at early stages of the life cycle with an analysis of the relationship between inbreeding coefficients and germinability; and (3) to estimate the primary outcrossing rate, with the estimates obtained in goals 1 and 2.

Materials and Methods

Seeds were collected from three populations, which differed in population size and degree of fragmentation (Fig. 1, Table 1). In each locality, cones were collected by climbing trees separated usually at least 10–20 m from each other and distributed throughout the sampling area. The population of Chiquihuitlán consisted of approx. 4000 reproductive individuals distributed in one 10 ha forest fragment and tiny

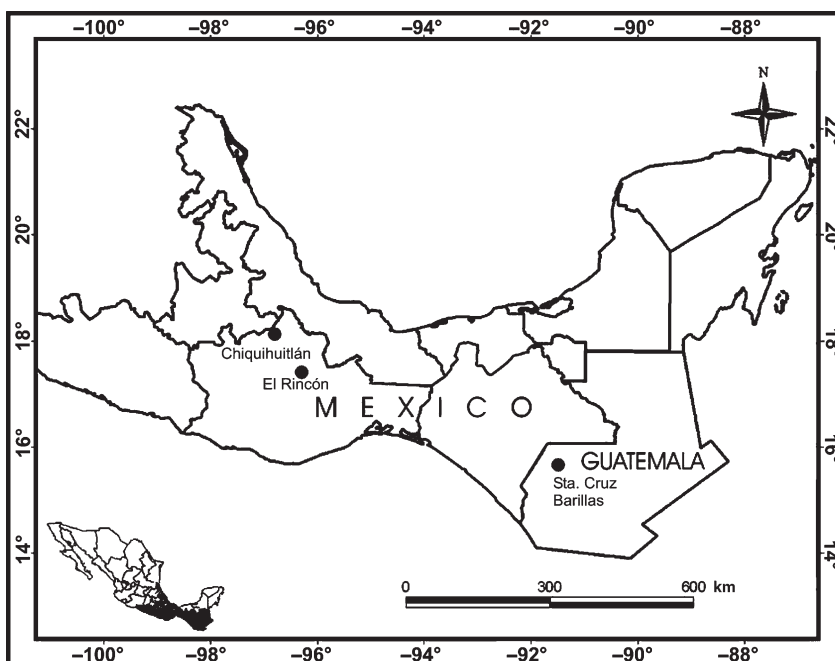


Fig. 1 Map of Mexico and Guatemala showing the location of the *Pinus chiapensis* populations investigated in this study. (Map by Raúl Rivera García.)

Table 1 Details of the populations of *Pinus chiapensis* used in this study, including population name, state (Mexico) or department (Guatemala), geographic coordinates, altitude, mean diameter at breast height (d.b.h.) and mean per-progeny germination rates

Population	State/ Department*	Altitude (m)	Latitude (N)	Longitude (W)	Mean d.b.h. (cm)	Mean per-progeny germination rate (% ± SE)
Chiquihuitlán	Oaxaca	1162	18°00′	96°46′	48.0	42.1 (5.9)
El Rincón	Oaxaca	1737	17°21′	96°18′	28.1	36.1 (2.2)
Barillas	Huehuetenango*	1683	15°47′	91°19′	41.6	26.9 (3.2)

forest patches, surrounded by a matrix of pasturelands and agricultural fields. Recruitment was detected. The Barillas population was larger: *c.* 40 000 adult individuals. *Pinus chiapensis* (Mart.) Andresen dominated the forest, distributed in 10–100 ha forest fragments and small patches, surrounded by agricultural lands. Recruitment was rare. El Rincon was the largest population (> 50 000 adult individuals) distributed in < 1500 ha of secondary forests dominated by *P. chiapensis*, which resulted from abandoning of corn-cropping areas. Tropical montane cloud forest was the original vegetation (Cordova & del Castillo, 2001; Bautista-Cruz & del Castillo, 2005). Regeneration was abundant in recently abandoned open areas.

Allozyme analyses

Allozyme studies were conducted on seedlings from fresh seeds, that were less than 4-months old germinated on moist filter paper at 24°C. Seed radicles were ground in 0.2 M phosphate buffer pH 7.5 (Conkle *et al.*, 1982). Extracts were adsorbed onto Whatman 4 mm chromatographic paper wicks. Samples were loaded on 12% starch gels. Aspartate amino transferase (AAT, E.C. 2.6.1.1) and glucose-6-phosphate isomerase (GPI, E.C. 5.3.1.9) were resolved on tris-citrate/LiB (pH 8.3) gels that run for 6–8 h at 75 mA (Conkle *et al.*, 1982). Phosphoglucumutase (PGM, E.C. 5.4.2.2) was resolved on histidine-citrate, pH 7.0 that run for 5–6 h at 50 mA (Cheliak & Pitel, 1984). Staining protocols for GPI, PGM and AAT followed Conkle *et al.* (1982); Cheliak & Pitel (1984) and Levy (1989), respectively. We used a variable number of seeds and maternal plants reflecting between-population variation in number of maternal trees available for sampling. Where several zones of activity were observed for a single enzyme, numerals following the enzyme abbreviation were used for identification. Band interpretation was based on segregation patterns observed in megagametophytes (Ramírez Toro, 2005), a haploid seed reserve tissue in conifers, and on the overall conservation of isozyme subunits and isozyme numbers in plants (Gottlieb, 1981; Wendel & Weeden, 1989).

Data analysis

Mating systems were analysed using Ritland's (2002) multilocus approach and software, which uses progeny arrays to calculate

multilocus (t_m) and single locus (t_s) outcrossing rates, the parental F estimate, the extent that siblings share the same father (correlation of paternity) and the extent of variation among arrays for selfing rate (correlation of selfing). Multilocus outcrossing rate estimation assumes that the loci studied are unlinked (Ritland, 2002). In *P. chiapensis* estimates of linkage disequilibrium were not significant for any pair of the above loci (R. F. del Castillo unpublished). Outcrossing rates were estimated from progeny genotypes with the Newton–Raphson method. Standard errors were calculated by 1000 bootstraps using the progeny array as resampling unit. Biparental inbreeding was tested by analysing the correlations of t_s among loci, r_s . The $1 - r_s$ values are indicative of the fraction of biparental inbreeding over the total inbreeding detected and tend to be less biased than the traditional $t_m - t_s$ used for biparental inbreeding assessment (Ritland, 2002).

Inbreeding depression from mating to seedling stage may bias our mating system estimates, which were based on analyses of seedlings. To correct for such a bias, we used Eqn 1, with the following estimate of inbreeding depression:

$$\delta = 1 - W_s/W_o$$

(W_s and W_o are the viability of the self- and outcross seeds, respectively). Seed viability was estimated from the proportion of germinated seeds out of the total number of seeds attempted to germinate per progeny, usually 100 seeds, as already described. Ungerminated seeds had an empty or necrotic embryo when dissected and no detectable enzymatic activity. W_s and W_o were obtained from linear regression of per-progeny seed viability on per-progeny F -values, estimated from the isozyme analyses described earlier. A total of 50 progenies, for which we have both germination and isozyme data, were used in this analysis. For prediction purposes, no transformation was applied since untransformed data gave better fit and better (more homogenous) residual behavior than transformed data (angular and log odds), recommended for proportions (Draper & Smith, 1981) or log transformation used for inbreeding depression analysis (Charlesworth & Charlesworth, 1987). A lack-of-fit analysis was performed to test the adequacy of the model (Draper & Smith, 1981). W_o and W_s were estimated from the values predicted at $F = 0$ and $F = 0.5$, respectively. The latter value is the expected F of self progenies from parents that were themselves product of random outcrossing,

Table 2 Mating system analysis of three populations of *Pinus chiapensis*: parameter estimates (mean \pm SD) and sample sizes

Population	Minimum variance parental F estimates	t_m	Minimum variance mean of t_s	Correlation of t_s values among families	Multilocus correlation of paternity estimates	Correlation of t_s values among loci	Families	Mean number of individuals/progeny
Barillas	-0.20 (0.064)	0.92 (0.094)	0.95 (0.096)	0.99 (0.475)	0.05 (0.077)	0.15 (0.470)	11	12.4
Chiquihuitlán	-0.20 (0.351)	1.20 (0.047)	0.82 (0.190)	-0.20 (0.054)	-0.20 (0.091)	0.00 (0.446)	8	7.5
El Rincón	-0.14 (0.084)	0.54 (0.057)	0.51 (0.061)	0.96 (0.03)	0.05 (0.105)	0.75 (0.163)	40	13.5

Minimum variance parental F estimates, multilocus outcrossing rates (t_m), minimum variance mean of single locus outcrossing rates (t_s), correlation of outcrossing values among families, multilocus correlation of paternity estimates, correlation of single locus outcrossing values among loci, number of families analysed and mean number of individuals per progeny included in the assessment.

and was used in our analysis since the estimated F of the maternal plants from all studied populations did not reveal inbreeding (see the Results section).

To obtain an upper bound of the corrected outcrossing rate, we replaced the s_m and δ values from Maki's formula (Eqn 1) with the upper 95% bound estimates of the estimated multilocus selfing rate $s_m = 1 - t_m$, assuming a normal distribution, and an estimate of δ using the upper 95% predicted values of W_s and W_o . Similarly, a lower bound estimate of the corrected outcrossing was obtained with the 95% lower bound estimates of selfing, with a δ -value calculated from the lower 95% predicted values of W_s and W_o . The SAS system version 9.1.3 (proc GLM) was used for statistical analyses (SAS Institute, 1986).

Results

Analysis of uncorrected mating system estimates

We assayed a total of 735 seedlings from 59 maternal plants for mating system analysis. In all populations the minimum variance F estimates of parental trees were negative, and suggest an excess (Barillas) or equilibrium heterozygosity relative to Hardy–Weinberg expectations (Chiquihuitlán and El Rincón) (Table 2). Multilocus estimates of outcrossing, t_m , varied widely among populations. In Barillas and Chiquihuitlán inbreeding was not detected as significant, suggesting that outcrossing is the dominant type of mating. By contrast, the estimate for El Rincón was significantly different from 0 and 1, indicating that part of the progeny is derived from outcrossing and part from inbreeding. In this population, selfing appears to be the most important source of inbreeding since the minimum variance mean of single locus outcrossing estimate, t_s , was similar and not significantly different from the multilocus estimate and the correlation of t_s among loci was high and not significantly different from 1 (Ritland, 2002). Therefore, this population appears to have truly mixed mating system. Correlations of outcrossing among families were high and not significantly different from one in Barillas and El Rincón, suggesting that outcrossing rates are homogeneous among

maternal trees within populations. By contrast, in Chiquihuitlán correlations of outcrossing were very low and not significantly different from zero. Multilocus correlations of paternity among maternal sibships were near, and not significantly different from zero in all the studied populations. Thus, the probability that individuals within the same progeny share the same pollen donor is not different from that expected by chance.

Inbreeding depression and primary mating system estimates

We used a single regression model of per progeny seed viability, V , vs per-progeny F -values, for all populations studied, since the F by population interaction was not significant ($P = 0.30$). We found a highly significant negative relationship between V and the minimum variance mean of the F -values: $V = 0.34 - 0.13F$ ($P < 0.0001$, $r^2 = 0.30$). The lack-of-fit test was not significant ($P = 0.79$), suggesting that there are no reasons to doubt of the adequacy of the model. Wetz's criterion for model adequacy (93; Draper & Smith, 1981), is also met, as the F -ratio of the regression model (20.16) exceeds more than four times the corresponding nominal F ratio (4.03) at $\alpha = 0.05$ for 1 and 48 df. Our model indicates that selfing on a plant derived itself from outcrossed parents decreases seedling viability in 19%, on average, that is, $\delta = 0.19$. However, among-progeny variation in inbreeding depression is large (Fig. 2). However, the slope of the line relating \log (germination) with F , indicates that the populations contain 0.88 diploid lethal equivalents per individual related with seed viability, but the fit of the line is poorer than the regression using untransformed data ($r^2 = 0.15$, $P = 0.006$). Figure 3 shows the corrected and secondary values of outcrossing rates per population. The corrected values of outcrossing were equal (Chiquihuitlán) or very similar (Barillas) to the secondary outcrossing estimates since selfing is nil or negligible in these populations. By contrast, the corrected outcrossing estimate for El Rincón was 10% lower than the secondary outcrossing estimate ($t_m = 0.54$, uncorrected; $t_m = 0.49$, corrected). Thus, correction of outcrossing estimates with inbreeding depression increased the variability of t_m values even more among populations.

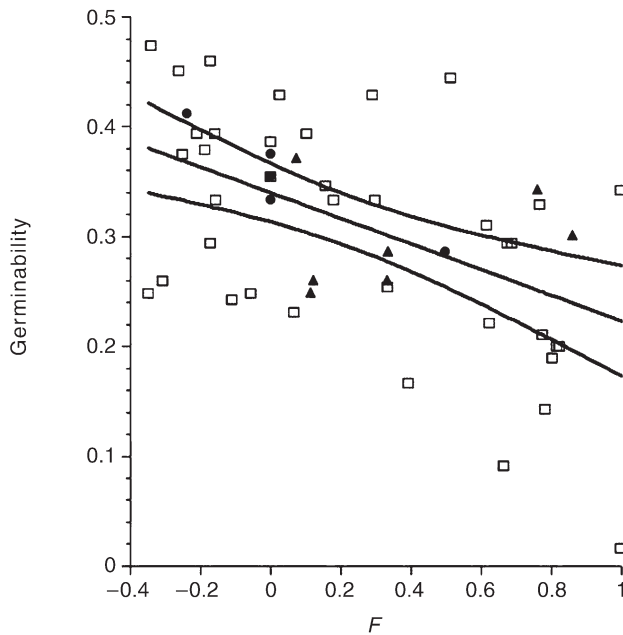


Fig. 2 The relationship between minimum variance means of progeny F -values and per-progeny germination rates in Chiquihuitlán (circles), Santa Cruz Barillas (triangles), and El Rincón (open squares) *Pinus chiapensis* populations. The least squares fitted line and 95% confidence intervals for the true mean value of germinability for a given F -value are also shown.

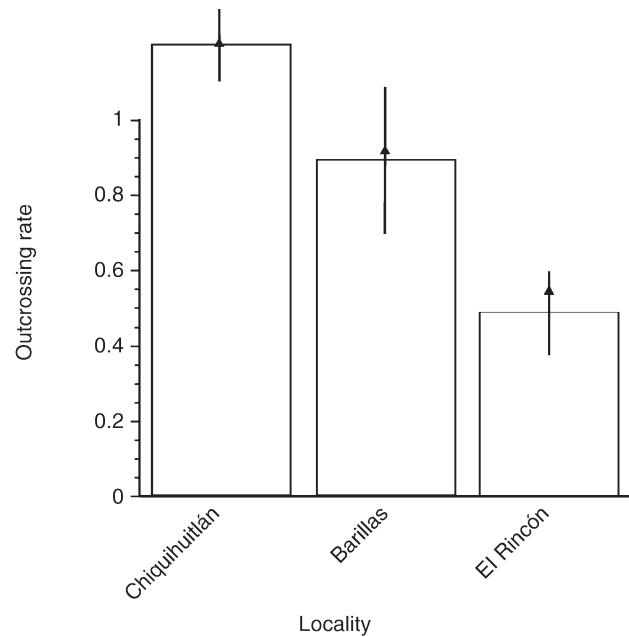


Fig. 3 Uncorrected (triangles) and corrected (bars) outcrossing rates of three populations of *Pinus chiapensis*. Error lines represent confidence intervals obtained from 95% confidence intervals of outcrossing estimates and 95% confidence intervals of inbreeding depression as explained in the Materials and Methods section.

Also, the correction for inbreeding depression in the t_m estimate for El Rincón increased more than twice the confidence interval. Nevertheless, our results still show significant differences between El Rincón estimate and those of the other two populations studied.

Discussion

Among-population differences in breeding system

This study reveals that variation of multilocus outcrossing rates values, t_m , among populations within the same species can be wide for neotropical trees. Indeed, to our knowledge, the range of variation of t_m values is the highest reported for *Pinus* even using uncorrected (secondary) t_m values. Reported outcrossing rates in *Pinus* are usually high, averaging 0.9 (Schemske & Lande, 1985; Delgado *et al.*, 2002). The few studies available for tropical pines report relatively high t_m values, similar to above figure, and with a narrow range: *Pinus caribaea* (0.85–0.92) and *Pinus oocarpa* (0.81–0.96) (Matheson *et al.*, 1989). The exception is the tropical pine *Pinus kesiya* with a broader t_m range (0.68–0.97; Boyle *et al.*, 1991). The estimate of t_m for El Rincón population is lower than the lowest reported for *Pinus* (Delgado *et al.*, 2002). Thus, variation in outcrossing rates among populations within the same species may be quite high at least in *Pinus*.

The high level of inbreeding detected at El Rincón population is surprising since this population is less fragmented and, at least, an order of magnitude larger than the other two populations studied, where outcrossing prevails. This suggests that factors other than small population size and fragmentation play an important role in reducing t_m values in *P. chiapensis*. Other studies have reported variation in the outcrossing rate among populations of the same species for which ecological and genetic explanations have been provided (Kesseli & Jain, 1985). Tolerance to inbreeding may increase in populations experiencing high inbreeding levels by purging of recessive deleterious alleles. In the Scots pine, the increased levels of selfing in some populations may have decreased the magnitude of inbreeding depression (Kärkkäinen *et al.*, 1996). We have not detected significant differences in inbreeding depression among populations. Moreover, our regression analysis suggests high variation in inbreeding depression levels within population – something common in other species of plants (del Castillo, 1998).

Landscape and vegetation structure differences among the studied population sites may help explain the differences in outcrossing values observed in *P. chiapensis* populations. The secondary forests at El Rincón are composed of a canopy layer of *P. chiapensis* trees and one or two emergent strata of shade-tolerant species most likely derived from neighboring primary or old growth forests (del Castillo & Blanco-Macías, 2007).

By contrast, the subcanopy layer in Barillas and Chiquihuitán forests is simpler and sparse presumably owing to the absence of neighboring primary forests as seed sources of shade-tolerant species and by the presence of cattle, which may inhibit plant recruitment and decrease growth in established plants (R. F. del Castillo, pers. obs.). The dispersive capacity of the atmosphere declines sharply with complex vegetation structure, which decrease wind velocity and increases turbulence (Whitehead, 1983). We therefore, hypothesize that a dense subcanopy layer at El Rincón secondary forests may decrease wind velocity and therefore, reduce pollen dispersability, explaining the low t_m detected in this population. By contrast, a thin subcanopy layer and fragmentation may favor outcrossing in Chiquihuitán and Barillas.

Correlation in paternity was low in all populations, including Chiquihuitán, which is relatively small and fragmented. This suggests that even in this population the number of potential parents per tree is high and outcrossing is random. Pollen production in pine is copious, and wind-dispersed pine pollen is well-known to travel long distances (Latta *et al.*, 1998; Ledig, 1998). These pollen features may help to reduce the effects of small population size and fragmentation in the mating system of *P. chiapensis*. High pollen flow may also help explain the low biparental inbreeding detected in *P. chiapensis* and appears to be a common trend for other neotropical tree species (Hamrick, 1994). Further evidence indicating that an important proportion of pollen movement occurs over relatively long distances in *P. chiapensis* is the small contribution of within-population spatial structure to inbreeding (Ramírez Toro, 2005). However, in other species of pine, biparental inbreeding is the most important source of inbreeding (El-Kassaby *et al.*, 1987; Fu *et al.*, 1992).

Inbreeding depression and its impact on outcrossing estimates

According with the F estimates, the frequency of heterozygosity of reproductive trees in *P. chiapensis* is equal or higher than that predicted by Hardy–Weinberg expectation. By contrast, a highly significant deficit of heterozygosity was found at seed stage in a previous study on the same species (R. F. del Castillo *et al.*, unpublished). This result and the positive association between per-progeny germination rates and per-progeny average heterozygosity frequency suggest that selection against homozygous individuals at different stages of the life cycle counterbalance any inbreeding resulting from population subdivision or consanguinity in *P. chiapensis*. Thus, self-progeny contribute little or nothing to reproduction. Inbreeding depression may also account for the value of Chiquihuitán greater than $t_s = 1$. Our results therefore are consistent with studies on other species of pine showing high inbreeding depression levels (Kärkkäinen *et al.*, 1996; Sorensen, 1999; Ledig *et al.*, 2001; Politov *et al.*, 2006, but see Fowler, 1965). The relatively high outcrossing levels detected in *Pinus* should

enhance the accumulation of deleterious recessive genes, which may explain the high inbreeding depression levels detected. The elevated inbreeding depression levels detected suggest that smaller and more isolated populations than those included in the present study can be vulnerable to extinction. Indeed, preliminary results have shown very low rates of outcrossing in a population of only 35 individuals (R. F. del Castillo & S. Trujillo, unpublished).

Our results indicate that inbreeding depression may affect mating system estimates even if progeny analyses are performed relatively early during the life cycle. Moreover, ignoring inbreeding depression may result in incorrect comparisons of mating systems among populations, even within the same species, as inbreeding depression does not affect evenly mating system estimates. Since inbreeding depression appears to be ubiquitous in plants, it is surprising that little attention has been paid to this potentially important source of bias in mating systems studies. Ideally, inbreeding depression should be assessed from independent data sets derived from controlled crosses, analysis of which includes effects of inbreeding at seed set stage. Our approach does not consider this early stage of the life cycle and therefore it is likely that our correction underestimates inbreeding depression. However, when direct approaches are precluded as in the case of tall trees, regression analysis between progeny F and progeny fitness estimates is a feasible option, which can provide a closer estimate.

The distribution of outcrossing rates among plant species have been subject to much attention in particular after the influential study of Lande & Schemske (1985) predicting a bimodal distribution. Several works have pinpointed sources of bias in empirical studies testing such predictions (Ugic & Kohn, 2006). Here we illustrate two additional problems. First, the distribution of outcrossing is traditionally investigated by assigning a single outcrossing category to each species analysed (Schemske & Lande, 1985; Vogler & Kalisz, 2001). The great variation in outcrossing rates observed in *P. chiapensis* populations indicates that classifying species on the basis of their t -value detected in a single or few populations can be misleading. Very little is known about the true range of variation in outcrossing rates among populations within species. Second, outcrossing rates used in empirical studies testing Lande and Schemske predictions are not usually corrected for inbreeding depression. In our study, the correction for inbreeding depression altered outcrossing rates by only 10% or less. Nonetheless, this distortion will operate unevenly among populations, making correct outcrossing estimates across populations difficult to achieve. Furthermore, correcting for inbreeding depression adds an extra source of random error to outcrossing estimates. Therefore, we may gain accuracy but not precision by including inbreeding depression in outcrossing estimation. In this regard, we provide additional information that supports the notion that conclusions of empirical studies analysing the frequency of mixed mating or the shape of the distribution of outcrossing rates in nature are

premature and a good comprehensive study would be very difficult to accomplish.

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