



Effect of patch size and isolation on mating patterns and seed production in an urban population of Chinese pine (*Pinus tabulaeformis* Carr.)

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ABSTRACT

Fragmentation is a critical issue for tree populations because the creation of small patches can reduce local population size and increase isolation, both of which can promote inbreeding and its negative consequences, as well as loss of genetic diversity. To test the hypothesis that patch size and isolation influence mating patterns or seed production in forest trees, we utilize the spatial array of trees of an urban population of Chinese Pine (*Pinus tabulaeformis* Carr.) planted in patches around Beijing. Our design includes 28 urban patches, with patch size ranging from 1 to 2000 adult trees and isolation (edge distance index) ranging from 37.5 m to 245.8 m. We examined the average number of seeds per cone and percentage of viable seed per cone for each patch as measures of seed production. By utilizing seven paternally inherited chloroplast microsatellite loci, we estimated the mating pattern parameters for each patch, including the level of selfing, the amount of immigrant pollen and the effective number of pollen sources (N_{ep}). Using a general linear model selection procedure based on AIC value, we found patch size was the best predictor of the selfing and immigration rate; smaller patches had a higher selfing and immigration rate. Small patches with one adult had relatively high N_{ep} which indicates connectivity among urban patches. However, due to the reduced amount of immigrant pollen and limited diversity of local pollen, intermediate sized patches (with 5–10 adults) had the lowest N_{ep} among the study patches. For patches with more than 10 adults, N_{ep} was increased with patch size. The percentage of viable seeds per cone significantly decreased with patch size, indicating a possible negative consequence of inbreeding. The effect of patch size on mating patterns and seed production suggests that the patches of trees experience less connectivity than trees within continuous forest. These findings indicate that forest management practices should emphasize the maintenance of an optimal patch size because, despite the fact that tree species show the potential for long distance pollen movement, the number of local trees strongly influences the mating patterns.

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1. Introduction

Fragmentation disrupts a continuous landscape into small and/or isolated patches, and may cause negative genetic and demographic consequences (Aguilar et al., 2008; Ellstrand and Elam, 1993; Holsinger, 1993; Ledig, 1992; Templeton et al., 1990; Young et al., 1996) by creating patches of small size and increased isolation from each other. These remnant populations may receive fewer migrants from elsewhere and local population size may be vulnerable to risk of loss of genetic variation (e.g. Bacles and Ennos, 2008; Fernandez-M and Sork, 2005; Hall et al., 1996; Hoebee et al., 2007; O'Connell et al., 2006; Robledo-Arnuncio et al., 2004).

Small patch size may promote selfing and mating with relatives in the local population and the resulting increased inbreeding can reduce subsequent reproductive success and fitness of the offspring (Barrett and Kohn, 1991; Ellstrand and Elam, 1993; Young et al., 1996). Isolated patches may experience genetic drift, inbreeding, and reduction in fitness due to the perturbation of the connectivity among populations (Sork and Smouse, 2006). Although tree populations may be resilient to these negative consequence because of individual longevity, high local genetic diversity, and extensive pollen flow (Hamrick, 2004), a recent meta-analysis of empirical studies of plant species (Aguilar et al., 2008) indicates that in the majority of cases, population fragments experience increased selfing, increased inbreeding depression, and decreased genetic diversity. The question is whether these impacts are due to a reduction in population size, an increase in isolation, or both.

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Mating patterns in plants, including selfing, bi-parental inbreeding, and outcrossing (Ritland, 2002; Shaw et al., 1981), as well as outcrossing with immigrant pollen, influences the risk of inbreeding depression and genetic drift (Ellstrand and Elam, 1993; Hall et al., 1996; Nason et al., 1999; Young et al., 1996). Patch size and isolation can negatively affect the mating patterns of forest tree populations (Aguilar et al., 2008; Eckert et al., 2010; Ellstrand and Elam, 1993; Sork and Smouse, 2006; Young et al., 1996). However, the effect varies greatly among studies. For example, increased selfing rate is found in small fragments of some species (e.g. *Sorbus torminalis* in Hoebee et al., 2007; *Pinus sylvestris* in Robledo-Arnuncio et al., 2004) but not others (e.g. *Picea glauca* in O'Connell et al., 2006). The relationship between immigrant pollen flow and fragmentation also shows inconsistent patterns – it is decreased in a small and isolated fragmented population of insect-pollinated *S. torminalis* fragments (Hoebee et al., 2007), but increased in fragmented populations of the wind-pollinated *Quercus humboldtii* (Fernandez-M and Sork, 2005). Other studies have also found increased immigration in patches, which suggests removal of intervening vegetation can promote gene flow (Bacles et al., 2005; Fore et al., 1992; Nielsen and Kjaer, 2010). These differences in response to fragmentation may be generally explained by various combinations of patch size and degrees of isolation (Holsinger, 1993; Sork and Smouse, 2006). Small and highly isolated patches, with limited local and immigrant pollen sources, are predicted to have the highest risk of loss of genetic diversity, while large and less isolated patches have the lowest risk (Sork and Smouse, 2006).

Seed production, denoted by the total number of seeds and number of viable seeds (Lee, 1988), is a key demographic parameter, and hence an important measure of early fitness of plants (Morgan, 1999). Fragmentation can lead to a reduction of seed production in plants, as a consequence of inbreeding depression in normally outcrossed populations (Ellstrand and Elam, 1993; Lee, 1988; Young et al., 1996). Indeed, experiments using hand-pollination of forest trees in various regions show that self-pollination events usually result in reduced seed production compared with outcrossing or open pollination events (Griffin and Lindgren, 1985; Seltsmann et al., 2009; Zhang, 2000). In natural populations, inbreeding in small and isolated fragments has led to reduce seed production (Hoebee et al., 2007; Robledo-Arnuncio et al., 2004). Thus, if fragmentation affects mating patterns, it can also negatively affect the population demographically through its impact on plant reproduction.

In this study, we examine pollen movement and seed production in Chinese pine (*Pinus tabulaeformis* Carr.), which is planted in small stands of trees throughout Beijing city. These urban populations include a range of habitat patches that vary in number of adults and degree of isolation from each other. Such stands provide a unique opportunity to test how patch size and isolation affect the selfing rate, the number of pollen donors, immigration, and seed production. Using chloroplast microsatellite genetic markers and ecological observations, we ask two questions: (1) what are the impacts of patch size, isolation, and their interaction on mating patterns of trees in these patches? (2) How do these factors influence seed production of these trees? To better answer the second question, a relatedness analysis was conducted to understand the potential influence of mating with close relatives on seed production. Moreover, environmental factors may potentially confound the analysis of mating patterns and seed production. For plant populations, the degree of openness of the canopy is critically important to the arrival of pollen (e.g. Mitchell, 2005) and to the production of seeds (e.g. Clark and Clark, 1987). Studies conducted in orchards of Chinese pine also show the effect of openness on pollen dispersal (Xu, 1993) and seed production (Zhang, 2000). Therefore, we include a separate analysis of this factor.

2. Materials and methods

2.1. Sample species and study site

Chinese Pine, *Pinus tabulaeformis* Carr. (Pinaceae), is a major tree species of northern coniferous forest in China (Wu, 1995; Xu, 1993). The species is monoecious, wind-pollinated, and predominantly outcrossing (97%, reported in Zhang et al., 2001). Pollen is shed from the male cone from the end of April to May depending on the temperature. Seeds mature in August of the next year and seed cones open in October. Chinese pine is one of the most common tree species in Beijing and its planting history can be dated back several 100 years. They are replanted mostly from natural populations near the city or from orchards around Beijing (personal communication with gardening workers), all of which represent the northeast type of the species (Xu, 1993). Planted trees in the urban landscape are mostly adults and can normally produce cones and seeds, with an average tree height of 4.65 m (± 1.11 m) and average diameter breast height (DBH) 19.17 cm (± 7.80 cm).

The area of Beijing is 16,807.8 km², with an urban area of 6338 km². This city is largely flat (85%) with a mean elevation of 47.3 m. Mean annual temperature is 13 °C with a January mean of -3.7 °C, a July mean of 25.2 °C and a mean annual average precipitation 507.7 mm (cf. http://www.gov.cn/test/2005-08/10/content_21501.htm). We conducted the study in the urban area, which comprises an approximate total area of 94.6 km² (Fig. 1).

2.2. Field methods

We selected 28 patches of Chinese pine trees that have been planted among a matrix of buildings (Fig. 1), which range in the number of adult trees (*Size*) present and degree of isolation (*Isolation*) (Table 1). The degree of isolation (*Isolation*) was calculated as the physical distance from one edge of a patch to edge of the nearest patch. A preliminary study of 95 urban patches indicated that seed production was variable for patches with less than 20 adults and less variable when patches larger than 20 adults. Moreover, a study of large continuous populations of Chinese pine indicated a low variance of selfing rate and paternity diversity among individuals and among sample sites (Wu, 2008). Hence, we sampled more small patches than patches larger than 20 adults (Table 1). Because the unit of analysis for our study is the patch, we only needed a few trees per patch to test our central hypotheses. Within each patch, we selected one to six trees and sampled 17–178 green closed cones during August and September 2007. To access seeds, individual cones were desiccated in the sun for 1 week to allow seed scales to fully open. For patches with less than 40 trees, we sampled leaf tissue and mapped all the individuals so that we could estimate immigration for the patch. For patches larger than 40, we only sampled the leaf tissue from seed donors and were unable to estimate immigrant pollen flow. For each cone, we categorized all seeds as either aborted or viable on color and weight of seeds. We calculated total seed number per cone (*Seeds#*) and percentage of viable seeds (*Fill%*) for each cone and used patch level averages of *Seeds#* and *Fill%* per tree per patch in our analyses.

2.3. DNA extraction and genotyping

Using a plant genomic DNA kit (Tiangen, Beijing, China), we extracted DNA from needle tissue and from 20 to 30 embryos per one to two trees per patch. The samples were genotyped using seven chloroplast microsatellites originally developed in *P. thunbergii* (Vendramin et al., 1996): Pt1254, Pt30204, Pt71936, Pt41093, Pt87268, Pt48210, Pt110048 (Table 2). Polymerase chain reaction (PCR) was conducted on PTC-200 (MJ Research Inc., Waltham, MA,

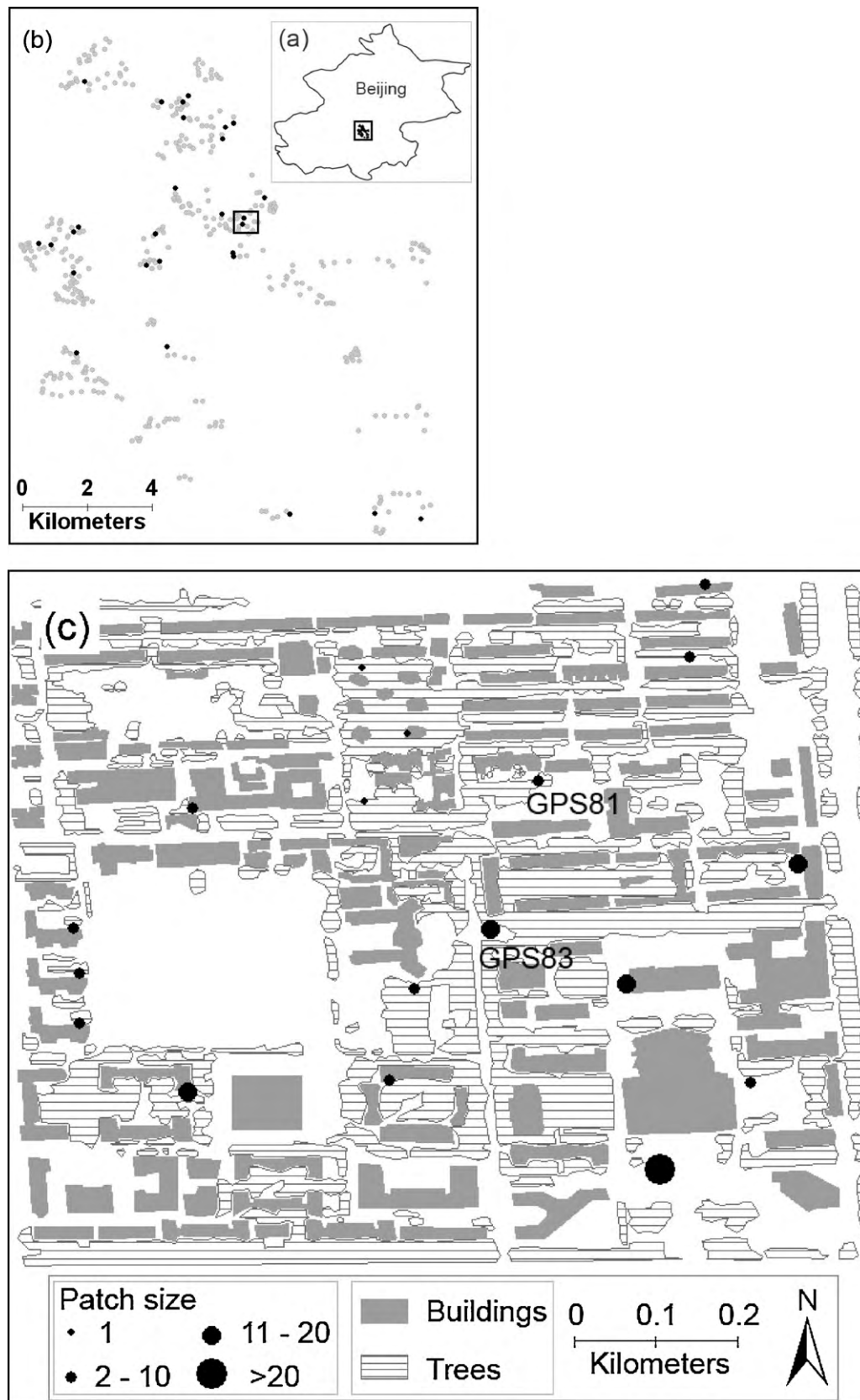


Fig. 1. (a) The focal patches in this study were mainly located in the urban area of Beijing (within the box). (b) Distribution of 28 focal patches (black dots) and its neighbor patches (grey dots). Note, not all patches had been located in the area, but for each focal patch, its nearest neighbor was clear. A detailed landscape of the box within (b) was described in (c). In (c), “Trees” is other vegetation in the urban landscape including *Sophora japonica* L., *Robinia pseudoacacia* L., *Ginkgo biloba* L., *Sabina chinensis* (L.) Antoine, *Juniperus formosana* Hayata, *Zizyphus jujuba* Mill. and etc.

Table 1
Characteristics of 28 focal patches sampled in an urban landscape of Beijing, China, including sample size for parameter estimation.

| ID | N | E | Size | Isolation | Cone trees# | Cones# | Paternity trees | Genotyped seeds | Openness measure |
|--------|--------|---------|------|-----------|-------------|--------|-----------------|----------------------|------------------|
| GPS289 | 39.947 | 116.314 | 1 | 53.0 | 1 | 33 | 1 | 24 | Y |
| GPS97 | 39.951 | 116.358 | 1 | 88.0 | 1 | 34 | 1 | 28 | Y |
| GPS352 | 40.000 | 116.317 | 1 | 104.8 | 1 | 36 | 1 | 28 | Y |
| GPS386 | 39.988 | 116.358 | 1 | 143.2 | 1 | 17 | 1 | 27 | Y |
| GPS295 | 39.926 | 116.339 | 1 | 245.8 | 1 | 29 | 1 | 30 | Y |
| GPS177 | 39.958 | 116.336 | 2 | 37.6 | 2 | 45 | 1 | 24 | – |
| GPS94 | 39.963 | 116.355 | 2 | 90.9 | 2 | 42 | 1 | 25 | Y |
| GPS403 | 39.996 | 116.345 | 2 | 151.8 | 1 | 22 | 1 | 29 | – |
| GPS105 | 39.968 | 116.367 | 2 | 174.3 | 2 | 34 | 1 | 20 | Y |
| GPS267 | 39.925 | 116.315 | 3 | 40.8 | 1 | 40 | 1 | 27 | Y |
| GPS199 | 39.960 | 116.315 | 3 | 61.3 | 3 | 69 | 1 | 24 | – |
| GPS213 | 39.970 | 116.342 | 3 | 96.1 | 2 | 52 | 1 | 22 | Y |
| GPS240 | 39.955 | 116.308 | 3 | 99.8 | 3 | 30 | 1 | 29 | Y |
| GPS241 | 39.955 | 116.304 | 3 | 142.2 | 3 | 45 | 1 | 26 | Y |
| GPS384 | 39.987 | 116.356 | 3 | 196.1 | 3 | 61 | 1 | 35 | Y |
| GPS81 | 39.962 | 116.361 | 4 | 82.9 | 3 | 84 | 1 | 26 | Y |
| GPS396 | 39.994 | 116.338 | 4 | 103.1 | 4 | 49 | 1 | 29 | Y |
| GPS98 | 39.952 | 116.358 | 4 | 112.2 | 4 | 97 | 1 | 26 | Y |
| GPS136 | 39.880 | 116.397 | 4 | 131.0 | 3 | 43 | 1 | 30 | Y |
| GPS10 | 39.880 | 116.373 | 6 | 118.2 | 3 | 77 | 1 | 30 | – |
| GPS374 | 39.984 | 116.355 | 8 | 95.7 | 4 | 67 | 1 | 27 | Y |
| GPS225 | 39.950 | 116.337 | 9 | 46.8 | 4 | 104 | 1 | 24 | – |
| GPS218 | 39.949 | 116.334 | 14 | 75.6 | 4 | 86 | 1 | 29 | – |
| GPS83 | 39.960 | 116.360 | 20 | 50.3 | 5 | 135 | 1 | 23 | Y |
| GPS399 | 39.994 | 116.344 | 39 | 45.6 | 4 | 85 | 2 | 30 + 29 ^a | Y |
| GPS389 | 39.990 | 116.344 | 80 | 81.6 | 3 | 75 | 1 | 29 | – |
| GPS185 | 39.958 | 116.314 | 110 | 64.2 | 3 | 76 | 2 | 29 + 29 ^a | – |
| GPS149 | 39.879 | 116.410 | 2000 | 240.1 | 6 | 178 | 2 | 29 + 29 ^a | Y |

ID, patch identity; N, northing; E, easting; Size, the number of individuals within the patch; Isolation, isolation index; Cone trees#, number of trees from which cones were collected; Cones#, number of cones collected for estimating seed production parameters; Paternity trees, number of trees from which seeds were used in paternity analysis; Genotyped seeds, number of seeds that had been genotyped; Openness measure, patches that had been measured patch openness (Y) or not (–).

^a Number of seeds that had been genotyped separately for the two sampled trees.

USA) in 20- μ l reactions consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each of four dNTPs, 0.8U Taq polymerase (TaKaRa Company, Tokyo, Japan), 4–10 ng of DNA template and 0.24 μ M of each forward and reverse primer (forward primers were labeled with fluorescent dye: 6-FAM, TAMRA, or HEX (Applied Biosystems)). PCR amplification was performed with the following touchdown procedure: 5 min at 95 °C, followed by 10 cycles of 1 min denaturing at 94 °C, 50 s annealing from 65 °C to 55 °C and 1 min extension at 72 °C followed by 15 cycles of 1 min denaturing at 94 °C, 50 s annealing at 55 °C and 1 min extension at 72 °C with a final extension step at 72 °C for 8 min. PCR products were separated on ABI-3100 sequencer by using a 50 cm capillary, polymerPOP-6TM (Applied Biosystems).

Fragment size was determined manually in GeneMapper software by comparison to size standard GeneScan-500 ROX. To evaluate the quality of genotyping, positive and negative controls were applied in each genotyping run. Allele size was assigned during two independent sessions to reduce scoring error. We re-genotyped individuals that showed discrepancies in those two reads. To estimate the rate of genotyping error, we re-genotyped 31 randomly chosen individuals.

Table 2
Motif, allele range, number of alleles and effective number of alleles (A_e) of the seven chloroplast microsatellites loci used in this study and originally developed in *P. thunbergii* (Vendramin et al., 1996).

| Locus | Motif | Allele range (bp) | Allele number | A_e |
|----------|---------------------------------|-------------------|---------------|-------|
| Pt30204 | C ₁₁ T ₁₂ | 139–151 | 10 | 3.29 |
| Pt71936 | A ₁₆ | 147–154 | 8 | 4.03 |
| Pt87268 | A ₁₁ | 159–171 | 9 | 2.43 |
| Pt110048 | T ₁₀ | 86–92 | 6 | 2.18 |
| Pt1254 | A ₁₁ | 63–82 | 18 | 6.85 |
| Pt41093 | A ₁₁ | 74–77 | 4 | 1.36 |
| Pt48210 | A ₁₂ | 87–91 | 5 | 2.20 |

2.4. Statistical analyses

2.4.1. Relatedness within patch

One of the advantages of this urban population for this study is that the local patches are unlikely to be related. To test this assumption, we calculated the extent of mating with relatives in urban pine patches using the program RELATEDNESS 5.0.8 (Goodnight and Queller, 1998) for each of 20 patches where all adults have been genotyped and patch size is larger than two trees (Table 1). To correct the potential bias of small sample size, we used a fixed background allele frequency from all genotyped adults in 28 patches. Standard errors of relatedness estimates were obtained by jackknifing over seven loci.

2.4.2. Paternity analysis

The theoretical probability of paternity exclusion based on the seven loci of chloroplast microsatellite was calculated through the formula of Jamieson and Taylor (1997). When calculating the exclusion probability, we treated the chloroplast genome as a single “locus” and each seven-locus haplotype as an “allele”.

To estimate the mating pattern parameters for each patch, we analyzed the paternity of all progeny using seven paternally inherited chloroplast genetic markers and simple exclusion methods. By comparing the haplotypes of progeny with that of all adults within that patch, progeny could be categorized as: (i) self-fertilized: only the seed source tree could not be excluded as the pollen parent; (ii) outcrossed within patch: only one tree (not the seed source) within the patch could not be excluded; (iii) immigrant: no pollen source could be assigned from trees within the patch; (iv) ambiguous: several trees within the patch could not be excluded. In one patch (GPS399), we found three trees with the same haplotype and thus the progeny that were fertilized by one of these three trees could not be assigned to a single parent. Because these trees were in the same patch as the progeny and were not the seed source,

this ambiguity did not affect our ability to estimate the number of self-fertilized and immigrant progeny for that patch.

For each patch, we calculated several mating pattern variables. *Selfing* was calculated as the percentage of seeds that were self-fertilized and *Immigration* was the percentage of seeds that were sired by immigrant pollen. Local outcrossing was calculated as $(1 - \textit{Selfing} - \textit{Immigration})$. Using the equation of Smouse and Robledo-Arnuncio (2005), the probability of paternal identity (*PPI*) estimates the probability that two offsprings within the same mother came from the same father. Thus, it is a measure of the correlated matings within the pollen pool and can be translated into the effective number of pollen donors per tree through the equation, $N_{ep} = 1/PPI$ (Smouse and Robledo-Arnuncio, 2005). For patches where more than one tree was sampled, we calculated a patch level estimation of *PPI* (and N_{ep}) by averaging *PPI* (and N_{ep}) values over all mother trees. The advantage of *PPI* and N_{ep} is that they can be estimated for all trees, even when the paternal sources are not genotyped. For large patches (>40 trees) it was impossible to genotype all possible pollen donors, so we used the genotype of the seed source tree to estimate *Selfing*, *Outcrossing* (calculated as $1 - \textit{Selfing}$), *PPI*, and N_{ep} but not immigration.

To test whether simple exclusion analysis underestimated selfing or overestimated immigration, we reassessed the paternity analysis allowing for a mismatch at one locus. We recalculated the *PPI* and N_{ep} by treating two haplotypes as identical when they differed at one locus. We then re-ran our analyses to see whether our results were comparable.

2.5. General linear model and model selection

To identify the contribution of patch size and isolation on mating pattern parameters and seed production, we used a general linear model approach (McCullagh and Nelder, 1989) and selected the best model based on Akaike information criterion (AIC, Akaike, 1974). Before model selection, we tested the linear correlation between variables to find potential interactions between explanatory variables and other suggestive relationships.

Because nonsignificant correlations could be due to a nonlinear relationship, we examined the potential relationship between explanatory variables (*Size*, *Isolation*) and response variables (*Selfing*, *Immigration*, *PPI*, N_{ep} , *Fill%*, *Seeds#*) using a nonlinear least-squares transformation method in STATA 9 (StataCorp LP). For the response variable (y) and the explanatory variable (x), the method uses a regression model $y = \beta_0 + \beta_1 \cdot x^\alpha$, where β_0 is the constant item, β_1 describes the slope that the response variable (y) changes with the explanatory variable in a form of x^α and α determines the nonlinear shape. An iteration method was used to find the α that best fits the data. Only when the regression model was significant at the 5% level was the nonlinear relationship between y and x accepted. We only used regression models that made biological sense in the above process. For example, *Selfing* and *Immigration* will never go below zero and *Fill%* will never go above one. Hence, in the regression model, we set coefficient β_0 equal to 0 for *Selfing* and *Immigration*, and β_0 equal to 1 for *Fill%*. For explanatory variables that had no accepted nonlinear relationship with a response variable, their linear relationship was used in the following model selection procedure.

We began the model selection with explanatory variables *Size*, *Isolation* and their interaction term ($\textit{Size} \times \textit{Isolation}$). We used a maximum of three explanatory variables, which theoretically yields a total number of eight potential models, which is less than our sample size of 28 patches. Burnham and Anderson (2002) suggested that it is preferable to include a small number of explanatory variables in model selection to avoid spurious relationships or other statistical problems. Therefore, for each response variable, we tested all potential models in R, Version 2.7.2 (R Development

Table 3

Average relatedness for each of 20 patches. The relatedness estimator was based on). SE was the standard error of the estimators and calculated by jackknifing over seven chloroplast microsatellite loci.

| ID | Size | Relatedness | SE |
|--------|------|-------------|-------|
| GPS94 | 2 | -0.533 | 0.230 |
| GPS105 | 2 | 0.190 | 0.298 |
| GPS177 | 2 | -0.403 | 0.205 |
| GPS403 | 2 | 0.022 | 0.314 |
| GPS199 | 3 | 0.252 | 0.235 |
| GPS213 | 3 | -0.035 | 0.066 |
| GPS240 | 3 | 0.215 | 0.257 |
| GPS241 | 3 | 0.013 | 0.249 |
| GPS267 | 3 | -0.188 | 0.096 |
| GPS384 | 3 | -0.157 | 0.075 |
| GPS81 | 4 | -0.146 | 0.086 |
| GPS98 | 4 | -0.187 | 0.042 |
| GPS136 | 4 | 0.066 | 0.175 |
| GPS396 | 4 | 0.027 | 0.082 |
| GPS10 | 6 | 0.032 | 0.057 |
| GPS374 | 8 | 0.045 | 0.087 |
| GPS225 | 9 | 0.045 | 0.046 |
| GPS218 | 14 | -0.005 | 0.042 |
| GPS83 | 20 | -0.007 | 0.033 |
| GPS399 | 39 | -0.005 | 0.022 |

Core Team, 2008) and chose the one with the lowest AIC as the best model.

2.6. Patch openness effect

Patch openness, or the proportion of visible sky within a patch, is related to light availability, which may affect the seed development process and obscure any patterns caused by patch size and isolation. To clarify the effect of patch openness on mating pattern and seed production, we measured patch openness using hemisphere photography in 20 out of 28 patches (Table 1). For each patch, eight hemisphere photographs were taken during May on a Nikon D100 with a Nikon 10.5 mm f/2.8 DX fisheye lens. The lens was placed on a tripod 1.6 m above ground and leveled for each photograph. Gap Light Analyzer (GLA) version 2.0 (Frazer et al., 1999) was used to calculate the openness (“%Cnpy Open” index in GLA) for each photograph and then patch openness (*Open%*) was calculated by averaging “%Cnpy Open” over eight photographs (Hill et al., 2006). Potential correlations between patch openness and patch characteristics, mating pattern and seed production were explored.

3. Results

3.1. Genotyping

We found 109 haplotypes among 152 adult trees and 201 haplotypes among 851 offspring. Within the 109 adult haplotypes, 78.0% occurred only once. The paternity exclusion probability for the seven-locus haplotypes was 97.21%. For the re-genotyped 31 randomly selected individuals (216 genotyping events across the seven loci), we found no discrepancy between the two independent genotyping processes, which indicated a low genotyping error (<0.5%). By allowing for mismatch at one locus, we kept 62 adult haplotypes and the paternity exclusion probability was 94.21%.

3.2. Relatedness within patches

We found that the mean level of relatedness of most of the patches was low, and varied from -0.533 to 0.066 (Table 3). Only three patches (GPS105, GPS199 and GPS240) showed relatively high relatedness (0.190 ± 0.298 ; 0.252 ± 0.235 ; 0.215 ± 0.257), which could lead to an overestimate of selfing, and *PPI*, and an underestimate of N_{ep} in these patches.

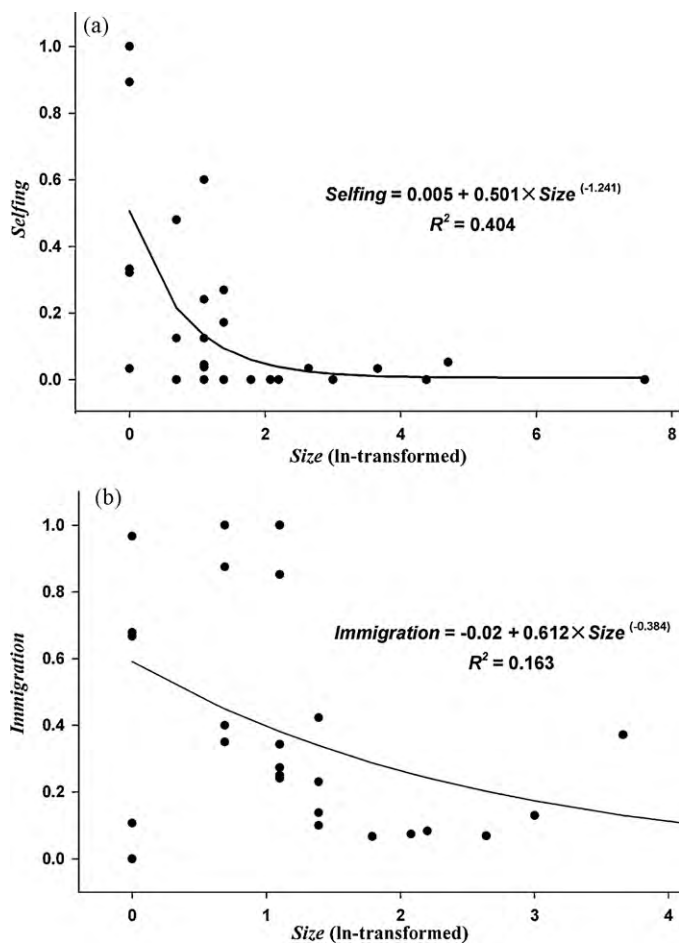


Fig. 2. Mating patterns as a function of patch size: (a) *Selfing*; (b) *Immigration* with patch size (*Size*). Black dots represent individual patches and the line is the predicted value based on the best model. Mating pattern parameters estimation was based on simple exclusion with seven chloroplast microsatellite loci.

3.3. Mating pattern

Among the 28 patches, *Selfing* and *Immigration* varied widely. *Selfing* varied from 0.00 to 1.00, with a mean selfing rate of 0.18 ± 0.27 ; *Immigration* varied from 0.00 to 1.00, with a mean of 0.39 ± 0.33 .

Size had negative exponential relationships with both *Selfing* and *Immigration* (Fig. 2a, b). In the model selection procedure, we used the nonlinear form of patch size for both of the response variables *Selfing* and *Immigration*. For *Selfing*, the transformed *Size* ($Size_S$) equaled to $Size^{(-1.241)}$ and for *Immigration*, the transformed *Size* ($Size_I$) equaled to $Size^{(-0.384)}$. We did not find any accepted nonlinear relationship between *Isolation* and the mating pattern parameters, nor between *Size* and *PPI* (or N_{ep}), so we used their linear form in the model selection procedure.

Patch size was positively correlated with *isolation* ($\rho = 0.452$, $P = 0.016$, $N = 28$, Table 4). However, we retained both variables in the model selection because their correlation was less than 0.7 and their separate contributions could be identified.

The best models for *Selfing* and *Immigration* included transformed *Size* ($Size_S$ and $Size_I$ separately) only. With increased patch size, both *Selfing* and *Immigration* decreased following a negative exponential function (Fig. 2). Small patches had a high proportion of selfing and immigration. However, for patches with more than five individuals, the mating pattern was dominated by local outcrossing (>80%, Fig. 3) and the level of selfing was low (less than 0.06).

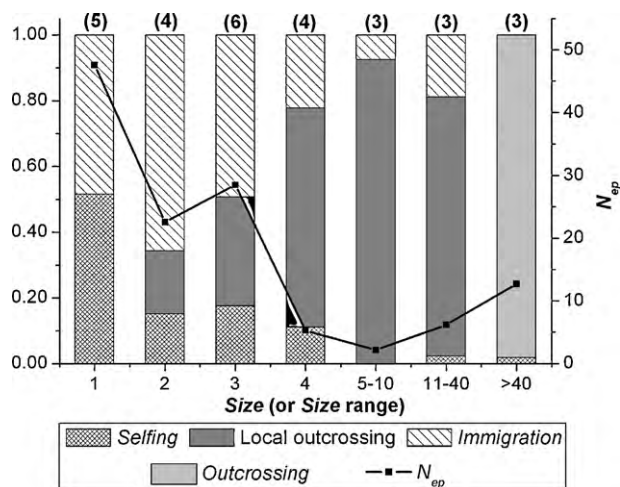


Fig. 3. Mean proportion of selfing, local outcrossing and immigration against patch size, averaged over 3–6 patches. Numbers above each bar are the number of sample patches of each patch size group. All sampled seeds within patch were assigned as selfing, local outcrossing and immigration, and total 1.00. For patch size >40, local outcrossing and immigration are pooled because they could not be distinguished. The line denotes the effective number of pollen donors.

The probability of paternity identity (*PPI*) varied from 0.005 to 1, with a mean of 0.28 ± 0.26 ; the effective number of pollen donors (N_{ep}) varied from 1 to 217.5, with a mean of 21.2 ± 45.3 . Most patches had low N_{ep} values (less than 20). Four small patches had a relatively high N_{ep} value, where N_{ep} in patch GPS295 was 217.5, in patch GPS241 was 108.3, in patch GPS177 was 69 and in patch GPS267 was 50.1. N_{ep} had a significant positive relationship with patch isolation (coefficient of *Isolation* in the best model of N_{ep} : 0.325, $P = 0.034$, Table 5). However, for *PPI* we did not find any correlation with the explanatory variables *Size* and *Isolation* (Table 5).

The effective number of pollen donors (N_{ep}) was positively correlated with *Immigration* ($\rho = 0.671$, $P = 0.000$, $N = 25$) and negatively correlated with local outcrossing ($\rho = -0.423$, $P = 0.035$, $N = 25$, Table 4). Patches with one adult had an average N_{ep} of 47.6, where a large proportion of offspring was sired from immigrant pollen (mean *Immigration* = 48.4%). N_{ep} decreased quickly as patch size increased from two to five trees, where local outcrossing increased from 19.3% to 66.7%. When patch size was 5–10 trees, the patches were dominated by local outcrossing (mean = 92.5%), N_{ep} was very low (mean = 2.2). For patches with more than 10 adults, N_{ep} was gradually increased as patch size increased (Fig. 3).

Except for N_{ep} , there was no significant relationship between the degree of isolation and any of the mating pattern variables based on the model testing results (Table 5).

When we reassigned paternity allowing for mismatch at one locus, estimates of *Selfing* increased by 0.03 ± 0.07 and those of *PPI* by 0.03 ± 0.04 , while estimates of *Immigration* decreased by 0.04 ± 0.05 , and those of N_{ep} by 12.8 ± 37.0 . The assignments of paternity with one mismatch did not change the qualitative results of any of the models. Hence, although use of simple exclusion may create some under- and over-estimates of different parameters, the analysis of patch size and isolation in our regression models were not dramatically affected by the estimates.

3.4. Seed production

Seed production varied among the studied patches: the number of seeds per cone (*Seeds#*) varied from 3.52 to 40.58 (mean 18.86 ± 9.35) and the percentage of viable seed (*Fill%*) varied from 0.16 to 0.73 (mean 0.43 ± 0.17).

Table 4

Pearson's correlation matrix for patch characteristics, mating pattern parameters, seed production and patch openness. Below the diagonal is the Pearson correlation and above the diagonal is the *P* value for each pair of correlation calculations.

| | Size | Isolation | Fill% | Seeds# | Selfing | Immigration | Local outcrossing | PPI | N _{ep} | Open% |
|-------------------|--------------------|--------------------|---------------------|--------------------------|-----------------|------------------|-------------------|----------------|-----------------|-------|
| Size | – | 0.016 | 0.076 | 0.177 | 0.480 | 0.251 | 0.032 | 0.342 | 0.982 | 0.763 |
| Isolation | 0.452* | – | 0.084 | 0.818 | 0.700 | 0.358 | 0.201 | 0.761 | 0.034 | 0.790 |
| Fill% | 0.341 ⁺ | 0.332 ⁺ | – | 0.763 | 0.313 | 0.336 | 0.709 | 0.066 | 0.531 | 1.000 |
| Seeds# | 0.263 | –0.046 | 0.060 | – | 0.021 | 0.069 | 0.799 | 0.072 | 0.027 | 0.020 |
| Selfing | –0.139 | 0.076 | –0.198 | 0.434[†] | – | 0.254 | 0.006 | 0.003 | 0.297 | 0.018 |
| Immigration | –0.238 | 0.192 | 0.201 | –0.370 ⁺ | –0.237 | – | 0.000 | 0.000 | 0.000 | 0.515 |
| Local outcrossing | 0.430* | –0.265 | –0.078 | –0.054 | –0.535** | –0.694*** | – | 0.168 | 0.035 | 0.159 |
| PPI | –0.186 | –0.060 | –0.352 ⁺ | 0.346 ⁺ | 0.534** | –0.762*** | 0.285 | – | 0.019 | 0.223 |
| N _{ep} | –0.005 | 0.401* | 0.124 | –0.418* | –0.204 | 0.671*** | –0.423* | –0.441* | – | 0.884 |
| Open% | 0.072 | 0.064 | –0.000 | 0.516* | 0.524* | –0.159 | –0.336 | 0.285 | –0.035 | – |

* *P* < 0.05.

** *P* < 0.01.

*** *P* < 0.001.

⁺ *P* < 0.10.

Patch size had a negative exponential relationship with percentage of viable seeds (*Fill%*). Hence, we used the transformed *Size* ($Size_F = -Size^{(-0.091)}$) in the model selection for the response variable *Fill%*. There were no accepted nonlinear relationship between *Fill%* and *Isolation*, *Seeds#* and *Size*, *Seeds#* and *Isolation*, so the linear relationships were used in the model selection procedure.

The best model for *Fill%* included transformed *Size* and an interaction between *Size* and *Isolation* (Model1 in Table 5, AIC = –106.4,

*R*² = 0.366). Larger patches have more viable seeds than small patches (coefficient of *Size_F*: β = 0.886, *P* = 0.001). Only patches with more than 20 adult trees maintained more than 50% viable seed.

There was no effect of *Isolation* on *Fill%* in the best model, but there was a weak and marginal effect of *Size* on *Fill%* (coefficient of the interaction term: –0.001, *P* = 0.001, Table 5).

We did not find any significant effect of *Size* or *Isolation* on *Seeds#* (Table 5).

Table 5

The top three models that describe the relationships between *Size*, *Isolation* or their interaction *Size* × *Isolation*, and mating pattern and early fitness parameters as selected by AIC value. The mating pattern was estimated by using simple exclusion with seven chloroplast microsatellite loci. Models in bold indicated the best fit models with the lowest AIC.

| Response variables | Model | R ² | <i>P</i> (Model) | AIC | Explanatory variables | β | SE | <i>P</i> (β) |
|--------------------|---------------------------|----------------|------------------|---------------|-------------------------------------|----------------|---------------|--------------|
| <i>Selfing</i> | Model1 | 0.404 | 0.000 | –84.62 | Intercept | 0.005 | 0.056 | 0.925 |
| | | | | | Size_S[§] | 0.501 | 0.119 | 0.000 |
| | Model2 | 0.441 | 0.001 | –84.45 | Intercept | –0.001 | 0.056 | 0.988 |
| <i>Immigration</i> | Model1 | 0.163 | 0.045 | –56.84 | Intercept | –0.020 | 0.202 | 0.922 |
| | | | | | Size_I[†] | 0.612 | 0.289 | 0.045 |
| | Model2 | 0.177 | 0.117 | –55.27 | Intercept | 0.400 | 0.160 | 0.020 |
| <i>PPI</i> | Model1[‡] | – | – | –73.98 | Intercept | 0.283 | 0.049 | 0.000 |
| | Model1 | 0.161 | 0.035 | 211.65 | Intercept | –13.298 | 17.401 | 0.452 |
| | Model2 | 0.204 | 0.058 | 212.17 | Intercept | –20.029 | 18.219 | 0.282 |
| <i>Fill%</i> | Model1 | 0.366 | 0.003 | –106.4 | Intercept | 1.072 | 0.203 | 0.000 |
| | | | | | Size_F[¶] | 0.886 | 0.246 | 0.001 |
| | Model2 | 0.399 | 0.006 | –105.92 | Size_F × Isolation | –0.001 | 0.001 | 0.025 |
| <i>Seeds#</i> | Model1[‡] | – | – | 126.15 | Intercept | 18.864 | 1.767 | 0.000 |

§ Nonlinear transformation of *Size* for *Selfing*: $Size_S = Size^{(-1.241)}$.

† Nonlinear transformation of *Size* for *Immigration*: $Size_I = Size^{(-0.384)}$.

¶ Nonlinear transformation of *Size* for *Fill%*: $Size_F = -Size^{(-0.091)}$.

‡ Models with no significant variables but the intercept.

3.5. Patch openness

The proportion of visible sky of each patch (*Open%*) varied from 0.27 to 0.87, with a mean of 0.51 ± 0.15 . No correlation was found between *Open%* and patch size and isolation, but we found a significant relationship of patch openness with *Selfing* ($\rho = 0.524$, $P = 0.018$, $N = 20$) and *Seed#* ($\rho = 0.516$, $P = 0.020$, $N = 20$). *Seeds#* was also significantly correlated with *Selfing* ($\rho = 0.434$, $P = 0.021$, $N = 28$) and N_{ep} ($\rho = -0.422$, $P = 0.025$, $N = 28$) (Table 4).

4. Discussion

For our study species, patch size is a critical determinant of the level of selfing that occurs, the extent of immigration, and the proportion of seeds that mature within that patch. Small patches with less than four individuals have a high effective number of pollen donors (N_{ep}), due to the large contribution of immigrant pollen. However, these small patches have a higher selfing rate than big patches, which suggests a high risk of genetic drift and inbreeding depression for these small patches. For intermediate sized patches with 5–10 trees, limited local pollen source diversity results in a low effective number of pollen donors, even though the selfing rate is largely reduced. Big patches have a lower selfing rate, and have higher percentage of viable seed, which may be the result of less inbreeding depression. Immigration is found in most of the studied patches, which suggests that the patches show some degree of genetic connectivity via pollen flow. We did not find any relationship between the variation in isolation and any outcome variables except the effective number of pollen donors. Collectively, these results illustrate that the impact of patch size is a complex interplay between selfing, local outcrossing, and immigration, and this interplay shapes the risk of genetic drift or inbreeding depression.

For most patches in this study, the relatedness estimator is low, which means the planted population of urban pines has a low chance of mating with relatives. Hence, this study provides novel insights on the contribution of selfing to various fitness measures, because the effect of small patch size is not confounded by the effects of matings with relatives. Except in very small patches (less than five trees), we found relatively low selfing rates (<0.06) in Chinese pine, which is comparable to estimates based on studies of natural populations of *Pinus* (0.009–0.350, Scofield and Schultz, 2006) and a seed orchard study of Chinese pine (0.03, Zhang et al., 2001). Thus, the intermediate sized patches seem to have received sufficient amounts of outcrossed pollen to prevent selfing from occurring. The small patches, however, have few local pollen donors and high selfing rates, possibly because fertilization takes place before the arrival of immigrant pollen. This conclusion is supported by a pollen trap experiment conducted using these urban patches which found that pollen limitation was common in small patches (Wang et al., unpublished data). Most of the fragmentation studies analyzed by Aguilar et al. (2008) which found increased selfing in fragments could be explained by pollen limitation and a predominance of selfing in small populations.

The arrival of immigrant pollen to small patches provides a source of outcrossed pollen, and a greater likelihood of reproductive success, but it does not prevent inbreeding in small patches. The compensation function of immigration has been observed in many forest tree studies (e.g. Bittencourt and Sebbenn, 2007; Nason and Hamrick, 1997) and is thought to be proof of the resilience of forest trees to fragmentation (Hamrick, 2004). However, our study together with those empirical fragmentation studies, show that the maintenance of genetic diversity and reproductive success in small patches is strongly dependent on the amount of immigrant pollen received from outside the patch. This in turn depends on a number

of species' traits such as flowering phenology and spatial configuration (Aguilar et al., 2008; Manel et al., 2003). Thus, if small patches become so isolated that pollen immigration is reduced, they face an extremely high risk of reduced genetic diversity, reduced fitness, and possibly local extinction. This consequence has been observed in field studies of wind-pollinated species with potential for long distance pollen dispersal (e.g. Bacles and Ennos, 2008; Jump and Penuelas, 2006; Robledo-Arnuncio and Gil, 2005).

Local outcrossing seems to reduce the selfing rate, but it also seems to reduce the contribution of immigrant pollen, possibly because local pollen can swamp out immigrant pollen. Consequently, with both limited local pollen source diversity and reduced immigration, intermediate sized patches have extremely low N_{ep} (2.2). This finding is similar to that found in a study of *P. glauca* (O'Connell et al., 2006), where the effective number of pollen donors, not the selfing rate, was reduced in small patches of less than 10 individuals. In natural populations, where spatial genetic structure often exists, low availability of pollen donors would probably increase bi-parental inbreeding and may lead to inbreeding depression (Butcher et al., 2005; Fernandez-M and Sork, 2005). Meanwhile, a low effective number of pollen donors may reduce genetic diversity in offspring and cause long-term genetic effects within the patch (Young et al., 1996). Hence, regional populations comprised of intermediate sized patches with a sufficient number of local individuals to allow within patch outcrossing, yet prevent fertilization from immigrant pollen sources, may be at risk in the long run of reduced genetic diversity.

The positive relationship between the proportion of viable seeds and patch size provides evidence of the effect of high selfing rates in small patches. A lower number of viable seeds may indicate low individual reproductive fitness, which has also been observed in other forest tree fragments. For example, a much higher abortion rate accompanied with higher selfing rates was found in small fragments of *Pinus sylvestris* compared to continuous forest sites (Robledo-Arnuncio et al., 2004). However, the correlation between the measured selfing rate and the proportion of viable seed was not significant in this study, which may be due to the variation of inbreeding depression adjusted by patch openness (*Selfing* and *Open%*, $\rho = 0.524$, $P = 0.018$, Table 4). For a light loving species like Chinese pine, higher patch openness means more available light within the patch, which may reduce the abortion rate of selfed offspring (Armbruster and Reed, 2005; Cheptou, 2005) and thus more selfed offspring can survive to the filled seed stage to be measured. Nonetheless, the improvement does not change the trend of smaller patches having fewer viable seeds. Hence in natural forest, small populations with reduced reproductive success from high selfing rates and bi-parental inbreeding will face a potentially critical survival problem in the long term.

The positive relationship between the degree of isolation and the effective number of pollen donors (N_{ep}) indicates the extensive connectivity via pollen flow among those urban patches. The lack of association between the degree of isolation and the other mating variables also supports this apparent connectivity due to the long tailed pollen dispersal kernel, which is often found in wind-pollinated tree studies. For example, Robledo-Arnuncio and Gil (2005) found pollen of *P. sylvestris* traveled more than 30 km. Soil surface pollen concentration analysis in an urban landscape has shown *Pinus* pollen can disperse 10 km or further (Stuart et al., 2006). In Beijing, a 10 km neighborhood for a Chinese pine patch includes a large number of patches and thousands of trees (Fig. 1), which means extensive and diverse pollen could reach a focal patch. Hence, there may be no significant effect of isolation among the patches. Moreover, we found the contribution of pollen from nearest neighbor patches to be low. For example, out

of 12 focal patches where we had genotyped individuals in the nearest neighbor patches, only two patches had a proportion of immigrant pollen from the nearest neighbor, and the proportion was low.

All patches showed functional connectivity through pollen flow, but it would be a mistake to conclude that isolation does not affect mating patterns, because the impacts of size on these patches of trees are related to their fragmented nature, and would not be seen in a continuous forest. If this set of patches behaved like a “patchy population” (Hastings and Harrison, 1994), then we may not have seen the effect of patch size. It is possible that the variation in isolation between patches is sufficiently large to enhance the effect of local tree density but not sufficient to detect an impact of isolation. O’Connell et al. (2006) studied the effects of fragment size on the mating pattern and seed production of *P. glauca*. Patch size was not a significant variable in their study, and they concluded that there is enhanced pollen flow among fragments in the agricultural landscape. In the urban setting of our study, buildings may act to impede pollen movement, a hypothesis supported by pollen trap studies (Alcazar et al., 1999). Hence, it is possible that fragmentation can exacerbate the impact of local trees on pollen flow unless gene flow is enhanced by the landscape context.

The above findings have important implications for forest management. Most of the former fragmentation studies related to forest tree species are based on comparisons among few patches, which often lack statistical power or are not designed in a way as to separate the effect of different explanatory variables, such as patch size, isolation or other environment factors (McGarigal and Cushman, 2002). Hence, the extent to which forest tree species may be sensitive to fragmentation remains unclear. By utilizing a set of planted Chinese pine with minimal spatial genetic structure, we have demonstrated that patch size is a critical determinant of the genetic risk to fragmentation. When forest patches show some degree of isolation with other patches, small and intermediate sized patches will experience an increase in inbreeding and inbreeding depression, and those patches will face possible extirpation in the long run. Based on local matings only, big patches can maintain high genetic diversity, and thus they are less sensitive to isolation. Hence, for forest management, maintaining large patches is the lowest risk strategy for protecting trees against the negative consequence of fragmentation, while for small and intermediate sized patches, conservation efforts should be focused on the connectivity with other patches.

5. Conclusions

The mating patterns observed in this urban population of Chinese pine illustrate that the number of adults in the local patch critically influences the selfing rate, degree of immigrant pollen received in the patch, and number of viable seeds. We did not find a significant association between the variation in isolation and any of these variables, but these patches are sufficiently isolated that mating patterns are dominated by local processes. Despite the ability of long distance dispersal to retain connectivity among patches, which is common in wind-pollinated species, this study shows that the availability of local pollen sources can swamp out immigrant pollen contribution. Thus, these patches may not be isolated, but their reduced connectivity enhances the contribution of local pollen sources. For historically outcrossing populations, this patchiness puts very small and intermediate sized patches at increased risk of inbreeding depression and loss genetic diversity. This empirical study provides evidence that fragmented natural tree populations can be vulnerable to patch size and patchiness, despite potential for long distance gene flow.

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