Heredity (2007) 99, 580–591

www.nature.com/hdy

ORIGINAL ARTICLE

Patterns of pollen and seed dispersal in a small, fragmented population of the wind-pollinated tree *Araucaria angustifolia* in southern Brazil

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Paternity analysis based on eight microsatellite loci was used to investigate pollen and seed dispersal patterns of the dioecious wind-pollinated tree, *Araucaria angustifolia.* The study sites were a 5.4 ha isolated forest fragment and a small tree group situated 1.7 km away, located in Paranα State, Brazil. In the forest fragment, 121 males, 99 females, 66 seedlings and 92 juveniles were mapped and genotyped, together with 210 seeds. In the tree group, nine male and two female adults were mapped and genotyped, together with 20 seeds. Paternity analysis within the forest fragment indicated that at least 4% of the seeds, 3% of the seedlings and 7% of the juveniles were fertilized by pollen from trees in the adjacent group, and 6% of the seeds were fertilized by pollen from trees outside these stands. The average pollination distance within the forest fragment was 83 m; when the tree group was included the pollination distance was 2006 m. The average number of effective pollen donors was estimated as 12.6. Mother-trees within the fragment could be assigned to all seedlings and juveniles, suggesting an absence of seed immigration. The distance of seedlings and juveniles from their assigned mother-trees ranged from 0.35 to 291 m (with an average of 83 m). Significant spatial genetic structure among adult trees, seedlings, and juveniles was detected up to 50 m, indicating seed dispersal over a short distance. The effective pollination neighborhood ranged from 0.4 to 3.3 ha. The results suggest that seed dispersal is restricted but that there is long-distance pollen dispersal between the forest fragment and the tree group; thus, the two stands of trees are not isolated.
Heredity (2007) **99,** 580–591; doi:10.1038/sj.hdy.6801019; published online 10 October 2007

Keywords: *Araucaria angustifolia*; coniferous; microsatellite loci; spatial genetic structure; gene flow

Introduction

Genetic connectivity in populations of plants is determined by gene movements among them (Sork and Smouse, 2006). Gene flow in forest trees involves both pollen and seeds (Godoy and Jordano, 2001; Smouse and Sork, 2004). Male gametes are dispersed from the paternal to the maternal parent via pollen; in addition, embryos containing contributions from both parents are dispersed in the form of seeds (Hamrick *et al*., 1993). Patterns of pollen and seed dispersal greatly influence the genetic structure and effective size of plant populations (Adams, 1992; Dow and Ashley, 1998; Sousa and Hattemer, 2003). High levels of gene flow are expected to maintain genetic cohesion among populations, whereas low levels are thought to produce genetic differentiation through genetic drift and local selection.

Habitat fragmentation reduces areas of continuous forest to small, separate remnants and may decrease the effective size of tree populations (Cascante *et al*., 2002), disrupt the mating system and interrupt gene flow (Jump and Penuelas, 2006). When populations become genetically isolated, they are at risk of losing the genetic diversity that is critical to their long-term survival (Sork and Smouse, 2006). Forest fragmentation has been shown to produce an immediate loss of alleles that is associated with a reduction in population size (White *et al*., 1999; Jump and Penuelas, 2006). Additionally, mating systems and gene flow are disrupted, resulting in significantly elevated levels of inbreeding and population divergence and a reduction in genetic diversity within populations (Jump and Penuelas, 2006). However, the longevity of trees, combined with effective seed and pollen dispersal, can enhance their resistance to the negative effects of forest fragmentation (Hamrick, 2004; Jump and Penuelas, 2006). Thus, the key element for predicting the effects of fragmentation is the level of gene flow among populations (Hamrick, 2004).

Approaches based on microsatellite markers have been successfully used to estimate effective gene flow by comparing the segregation of genetic markers in parental and offspring cohorts (Lian *et al*., 2001; Burczyk *et al*., 2004a). Microsatellite markers provide a means of determining pollen donor patterns and level of gene flow through pollination and seed dispersal. The exclusion probability for paternity assignment is high owing to codominant inheritance and an elevated level of polymorphism (Dow and Ashley, 1998; Lian *et al*., 2001). The usual way to estimate pollen flow based on genetic markers is to use progeny arrays (seeds) sampled from individual seed-trees (Burczyk *et al*., 2004a). Movement

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Received 22 January 2007; revised 26 April 2007; accepted 23 May 2007; published online 10 October 2007

of successful pollen can then be traced by examining the relative locations of identified maternal and paternal trees. External gene flow from pollen can be estimated from the proportion of pollinations in which all trees in a stand are excluded from paternity (Dow and Ashley, 1998; Streiff *et al*., 1999). This procedure provides estimates of effective pollen flow observed at the seed stage only (Burczyk *et al*., 2004a). Complementary, realized pollen flow can be observed in the regeneration and juvenile stages if the locations of these individuals and all potential male and female parents within the local population are known and analyzed by genetic markers (Burczyk *et al*., 2004a). Seed dispersal is more difficult to analyze this way since the use of exclusion methods is limited when both parents are unknown; moreover, it is not possible to distinguish father and mother-trees in hermaphroditic and monoecious species (Dow and Ashley, 1996; Godoy and Jordano, 2001; Hamrick, 2004; Burczyk *et al*., 2006). In dioecious species, likelihood methods for inferring maternity (maternity analysis) are particularly powerful when a pattern of mating can be assumed, as in, for example, a random mating model (Schnabel *et al*., 1998). These species are sexually distinct, with the reproductive parts of individual plants being solely male or solely female. Thus, male and female parents can be distinguished using maximum likelihood paternity assignment (Meagher and Thompson, 1986) if a sufficiently high number of polymorphic loci and all adult male and female reproductive individuals are sampled. When both male and female parents are identified, pollen and seed dispersal distance can be assessed (Meagher and Thompson, 1987). Meagher and Thompson (1987) successfully used this method to identify male and female parents and to quantify pollen and seed dispersal in a population of the dioecious long-lived perennial species *Chamaelirium luteum* in the United States.

Finally, within-population gene movements have been addressed by analyses of spatial genetic structure at a microgeographic level (Gonzαlez-Martvnez *et al*., 2002; Hardy *et al*., 2004; Dutech *et al*., 2005; Jones and Hubbell, 2006).

We investigated patterns of pollen and seed dispersal in a small, isolated fragment of *Araucaria angustifolia* (Bert.) O. Ktze using eight nuclear microsatellite loci and applying a paternity likelihood approach. We also studied the effect of pollen immigration from a small, isolated group of 11 *A. angustifolia* trees located at a distance of 1.7 km from the fragment. We tested two main hypotheses. First, in comparison with many other wind-pollinated coniferous species, *A. angustifolia* pollen is relatively large (61.50 μm), non-saccate, has a reduced ability to float (12.02–18.98 cm/s), and reaches the ground much more rapidly (Sousa and Hattemer, 2003). These factors, combined with the typically high population densities of *Araucaria* forests, can limit pollen movement between and within populations and reduce the number of pollen donors contributing to the next generation (Sousa and Hattemer, 2003; Sousa *et al*., 2005). In addition, the forest fragment we studied appeared to be physically isolated, resulting in the forest stand and the tree group being genetically separated from each other and from further stands. Therefore, we tested the hypothesis that pollen flow distance would be reduced. Second, the seeds of *A. angustifolia* are heavy, and are primarily dispersed by autochory under the canopy of the seed-trees, although they can be secondarily dispersed by birds, rodents and other mammals (Carvalho, 2003). Autochory can produce spatial genetic structure and mating among relatives if pollen flow occurs over short distances. Therefore, we tested the hypothesis that spatial genetic structure would be present in the forest fragment.

Materials and methods

Studied species

A. angustifolia (Paranα pine) is a subtropical, wind-pollinated, dioecious, coniferous tree species endemic to South America. It is an economically important tree in southern Brazil (Sousa *et al*., 2003), providing raw material for the pulp and paper industry and timber for construction. The natural distribution of the species in Brazil ranges from 19°15′S (Minas Gerais State) to 31°30′S (Rio Grande do Sul State). It can be also found in small patches in Argentina and Paraguay. The species grows exclusively in the tropical wet mixed forest (*Araucaria* forest) in the Alluvial (gallery), sub-Montana, Montana and high-Montana mountain formations, between altitudes of 500 and 2300 m (Carvalho, 2003). In the early 1900s, this species dominated the forests of southern Brazil in long, continuous, dense areas, but subsequent intensive exploitation and policies encouraging clear-cutting (Sousa *et al*., 2005) have resulted in less than 3% of the original *Araucaria* forest remaining (Carvalho, 2003).

Study site

The study was conducted in a 5.4 ha forest fragment localized on a farm and a small group of 11 isolated trees (group) about 1.7 km distant from the fragment (Figure 1). The study area is located on the plateau of Paranα State within the Iguaηu catchments (latitude 25°57′S and longitude 52°11′W). Geologically, the area is formed from Triassic/Cretaceous volcanic (Trapp) flows of the Serra Geral mountain formation. The climate is transitional between subtropical (Cfa) and temperate (Cfb), according to the Koeppen classification. Average annual precipitation ranges from 1800 to 2100 mm and annual average temperature is 16–20°C. The natural vegetation of the region is *A. angustifolia* forest associated with broad-leaved species. Fragmentation in this area dates from 1920 to 1980 and the current landscape is a mosaic of patches of agricultural land, pasture, remnant steppe, urban areas and forest. The study fragment is surrounded by intensive agricultural activity and pasture, and there is no other *Araucaria* forest nearby (>4 km). However, it is possible to observe isolated *A. angustifolia* trees or small groups of trees in the landscape. For these reasons, the selected fragment provided a good model for a study of the extent of potential isolation of a fragmented *A. angustifolia* population. In the forest fragment, regeneration can be found, adult trees are within different ages, and it is reasonable to assume that all seedlings and juvenile individuals were produced after the fragmentation. Before 1980, this fragment was also exploited by selective logging. In the site there are 124 male and 104 female *A. angustifolia* trees, with densities of 22.96 males, 19.26 females and 42.22 trees/ha.

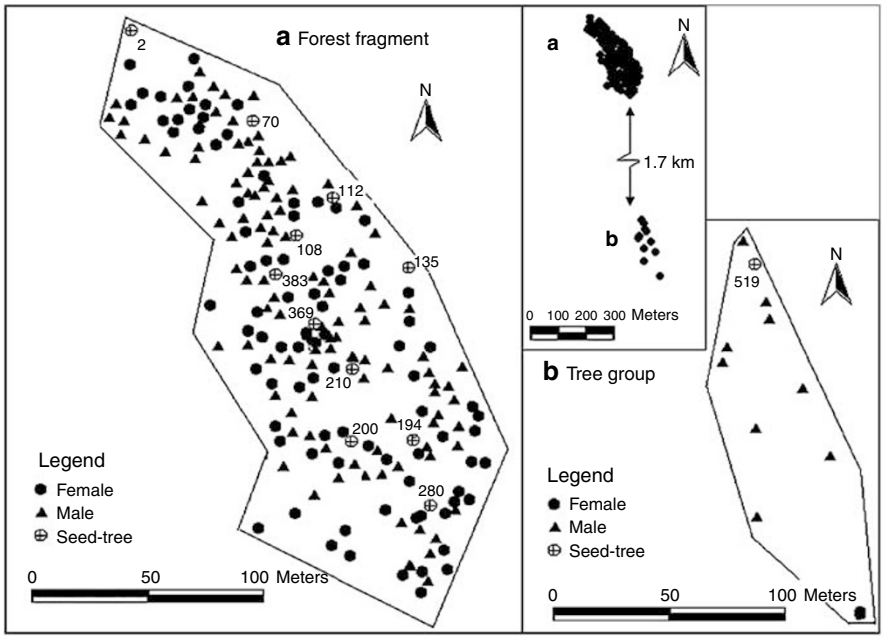

Figure 1 Map of *A. angustifolia* individuals in the analyzed forest fragment and the adjacent tree group.

Diameter at breast height (d.b.h.) ranges from 14.0 to 129.9 cm, height varies from 7 to 27 m and age was estimated to range between 46 and 250 years. Minimum distances between two males, two females, and a male and female are 3, 3.2 and 4 m, respectively. Maximum distances are 289.8, 334 and 331 m, respectively, and average distances are 98.4, 108.1 and 104.3 m, respectively. The nearest other *A. angustifolia* trees belong to a small group of 11 adults situated in a pasture. Nine of these trees are males and two are females. Their d.b.h. ranges from 40.7 to 82.8 cm, their height ranges between 8.5 and 14.5 m and their age was estimated to range between 70 and 160 years.

Sample collection

In the forest fragment, cambium was sampled from all 228 adult trees (124 males and 104 females). For three males and five females it was not possible to obtain DNA extractions sufficient for microsatellite locus amplification. Thus, the genetic analysis was based on 121 males and 99 females. In the group, all 11 trees were sampled. The distinction into female and male in both stands were made by direct observation of male and female cones. Leaf samples were collected from all seedlings and juveniles. In total, samples were taken from 66 seedlings and 92 juveniles from regenerations within the fragment. We defined seedlings as individuals less than 3 m tall (0.35–3 m). Generally, under natural conditions, *A. angustifolia* trees start reproduction at about 20 years of age (d.b.h. $\geqslant$20 cm, height $>$10 m), but early on the number of cones and male flowers is low. Thus, we defined trees without flowers, a d.b.h. $<$25 cm and a height between 3 and 10 m as juveniles. The spatial positions of all sampled individuals (adult, seedlings and juveniles) in both forest fragment and tree group were recorded. Seeds were randomly collected from the canopy of 12 seed-trees: 11 in the fragment, and 1 female from the group. From each seed-tree about 20 seeds were randomly collected from one to three cones. In the fragment, the minimum distance between a sampled seed-tree and a male was 6 m, the maximum was 331 m, and the average was 99.4 m. The minimum distance between seed-trees was 24.2 m, the maximum was 298.6 m and the average was 106.5 m. The cambium tissue was preserved in Eppendorf tubes with a solution of CTAB buffer (1/3) and ethanol (2/3) and stored at -20°C before DNA extraction. Leaf samples from juveniles and seedlings were stored in silica gel at room temperature before DNA extraction. Sampled seeds were stored at a temperature of -20°C before DNA extraction.

DNA extraction and microsatellite analysis

Total DNA was extracted from 220 adult trees, 66 seedlings, 92 juveniles and 230 seeds using the method of Mazza and Bittencourt (2000). DNA extraction from the seeds was adjusted slightly in that the megagametophyte (maternal origin) and embryo were extracted from 1 in every 20 open-pollinated seeds. The amplification and detection procedure for eight microsatellite loci are described in Salgueiro *et al*. (2005). The eight loci used were CRCAc2, Ag23, Ag62, Ag45, CRCAc1, Ag20, Ag56 and Ag94.

Genetic diversity

Genetic diversity for all sampled individuals from the forest fragment and from the group was characterized by the average number of alleles per locus (A), observed heterozygosity (H_o) and expected heterozygosity in Hardy–Weinberg equilibrium (H_e). The total paternity probability of the first ($Pr(Ex_1)$) and second parent ($Pr(Ex_2)$) was also estimated. These analyses were conducted using the program CERVUS 2.0 (Marshall *et al*., 1998).

Paternity analysis

Paternity analysis of each seed, seedling and juvenile was carried out using maximum-likelihood paternity assignment. Paternity was assigned by comparing

genotypes of adult males and seeds, females and males with seedlings and juveniles using the program CERVUS 2.0 (Marshall *et al*., 1998). To determine the putative pollen donor of the seeds, seedlings and juveniles, the analysis was performed using only male trees as parent candidates, since *A. angustifolia* is a dioecious species. To determine putative mother-trees, only reproductive female trees were used as parental candidates. Paternity or maternity was determined with base in Δ statistic (Marshall *et al*., 1998). To find the critical value of Δ for each confidence level in the paternity or maternity analyses, simulations were conducted using CERVUS 2.0. For these simulations we used 50 000 repetitions, with 0.01 as the proportion of loci mistyped, 95% as strict and 80% as relaxed confidence level and 80 individuals as probable male candidate parents for each seed-tree. From the paternity analysis, pollen dispersal distance was calculated for seeds, seedlings and juveniles based on the position of the seed-tree or probable mother-tree (for seedlings and juveniles), and putative pollen parent within the forest fragment and the tree group. To determine whether male mating success is a function of distance between males and seed-trees, we also compared the frequency distribution of effective pollinating male parents with the frequency distribution of the distances among all males and the 11 seed-trees in the forest fragment. This process was carried out using the Kolmogorov–Smirnov test (Sokal and Rohlf, 1995). The effective seed dispersal distance was calculated based on the positions of the seedlings and juveniles relative to their putative maternal parents.

Effective number of pollen donors, coancestry, inbreeding, variance effective size and effective neighborhood pollination area

The effective number of pollen donors (N_{ep}) of each seed-tree was estimated according to Burczyk *et al*. (1996) from the number of pollen donors (n_p) detected from paternity analysis results as $\hat{N}_{ep} = 1/\sum_{i=1}^{n_p} p_i^2$, where n_p is the number of embryos analyzed, p_i is the proportion of seeds sired by male *i*. From average over family effective number of pollen donors, we estimated the paternity correlation as $\hat{r}_p = 1/\hat{N}_{ep}$. On the basis of the paternity correlation, the average coancestry coefficient within families (Θ_{xy}) was estimated as $\hat{\Theta}_{xy} = 0.125(1 + \hat{F}_p)(1 + \hat{r}_p)$, where F_p is the inbreeding coefficient in the reproductive population (Sousa *et al*., 2005). The fixation index was estimated for adults (F_p), males (F_m), females (F_f), seeds (F_o), seedlings (F_s) and juveniles (F_j) as $\hat{F} = 1 - (\hat{H}_o/\hat{H}_e)$, where H_o is the observed heterozygosity, and H_e is the expected heterozygosity in Hardy–Weinberg equilibrium, using the GDA_NT program (Degen, 2006). The statistical significance of *F* values was tested using Monte–Carlo permutation methods (Degen *et al*., 1999). The variance effective size ($N_{e(v)}$) for families was estimated as $\hat{N}_{e(v)} = 0.5/\hat{\Theta}_{xy}$ (Cockerham, 1969). S.e. was used to determine the 95% confidence interval for the parameters. The effective population size of the reproductive population of the forest fragment was calculated using the Cockerham (1969) estimator as

$$\hat{N}_e = \frac{0.5}{\hat{\Theta}_{xy}((n-1)/n) + ((1+\hat{F}_p)/2n)},$$

where n is total number of individuals in the population, F_p is the inbreeding coefficient in the adult population, and Θ_{xy} is the group coancestry, calculated following the approach for dioecious species proposed by Lindgren and Mullin (1998):

$$\hat{\Theta}_{xy} = \frac{\sum_{x=1}^{n_f}\sum_{y\neq 1}^{n_f}\hat{\theta}_f}{4n_f^2} + \frac{\sum_{x=1}^{n_m}\sum_{y\neq 1}^{n_m}\hat{\theta}_m}{4n_m^2} + \frac{\sum_{x=1}^{n_f}\sum_{y=1}^{n_m}\hat{\theta}_{fm}}{2n_f n_m},$$

where θ_f, θ_m and θ_{fm} are the coancestry coefficients between females, males and males and females together, respectively, and n_f and n_m are the number of female and male adult trees. The coancestry coefficients were calculated using Loiselle's estimator (described below) and the SPAGEDI program (Hardy and Vekemans, 2002). The effective neighborhood pollination area (A_{ep}) was calculated for each seed-tree from the pollen-dispersal variance (σ^2), assuming a circular area around a central seed-tree, $\hat{A}_{ep} = 2\pi\hat{\sigma}^2$ (Levin, 1988).

Spatial genetic structure

Spatial genetic structure within the forest fragment was studied using the estimation of the average coancestry coefficient (θ_{xy}) between all pairs of adult trees, seedlings and juveniles. The θ_{xy} parameter measures the extent of relative similarity between individuals x and y relative to the mean genetic similarity between random individuals in the sample (Hardy and Vekemans, 2002). The coancestry coefficients were calculated using the estimator of Loiselle *et al*. (1995), which is defined for each *k*th allele at the *l*th locus in each pair of individuals, x and y, as

$$\hat{\theta}_{xy} = \left[\frac{\sum_l\sum_k (p_{xlk} - \bar{p}_{lk})(p_{ylk} - \bar{p}_{lk})}{\sum_l\sum_k \bar{p}_{lk}(1-\bar{p}_{lk})}\right] + \left[\sum_l \frac{1}{(2n_l - 1)}\right],$$

where p_{xlk} and p_{ylk} are the frequencies of allele k at the locus l in the individuals x and y, respectively (assuming values of 0, 0.5 and 1 in homozygote for alternative alleles, and heterozygote and homozygote individuals for the allele under consideration, respectively). $\bar{p}_{lk}$ is the average of the frequency of allele k at locus l in the sampled subpopulation, whereas n_l is the number of genes defined in the sample at locus l (number of individuals multiplied by the ploidy level minus the number of missing alleles). To visualize the spatial genetic structure, θ_{xy} values were averaged over a set of distance classes and plotted against the distances. We used 25 m distance intervals with a maximum distance of 250 m in each analysis. To test whether there was significant deviation from spatial genetic structure, the 95% confidence interval was calculated for each observed value and each distance class from 1000 permutations of individuals among locations. The confidence interval was used to construct the correlogram. These analyses were performed using the program SPAGEDI version 1.2 (Hardy and Vekemans, 2002). We also estimate the coancestry coefficients between seedlings and juveniles and their putative fathers and mothers assigned by paternity analysis. The expected coancestry coefficient for parent–sib relationships is 0.25.

Results

Paternity assignment

For the total sample, including all sampled individuals from the fragment (adults, juveniles, seedlings and seeds) and the group (adults and seeds), the number of alleles per locus ranged from 4 to 16 (average of 9.6 alleles), with a total of 77 alleles (Table 1). The observed heterozygosity ranged from 0.047 to 0.758 (average of 0.511), and the expected heterozygosity ranged from 0.062 to 0.863 (average of 0.596). The total paternity exclusion probabilities over eight loci of the first and second parent were 0.931 and 0.992, respectively.

Of the 210 seeds sampled in the forest fragment, pollen donors could be assigned for 189 (90%). Of the seeds sampled in the tree group, pollen donors were assigned for nine (4%, Table 2). Of these 198 seeds (189 + 9), 47 were assigned based on a confidence level of 95% and 142 on a confidence level of 80%. The other 12 seeds (6%) are likely to represent pollen that originated from trees outside the study sites or from the three male trees in the forest fragment that were not genotyped. We determined that, of the 121 potential pollen donors, 81 sired at least 1 seed. Of 20 seeds collected in the tree group from 1 female tree, pollen donors within the group could be assigned for 9 (45%); 11 seeds (55%) had pollen donors in the adjacent forest fragment. In the tree group, three males were assigned as fathers of the sample seeds and one of these males, located nearest (11 m) to the seed-tree, sired six of the nine seeds, according to the father assignment. Eleven different males from the forest fragment apparently sired the other seeds. Only one of these males is located in the northern part of the forest fragment. Seven males are located near the southern border of the fragment and three in the center (Figure 1a).

Mother-trees within the forest fragment were assigned to all 66 seedlings and 92 juveniles (Table 2). Of the 66 seedlings, nine were assigned based on a confidence level of 95% and 57 on a confidence level of 80%. Of the 92 juveniles, 15 were assigned based on a confidence level of 95% and 77 on a confidence level of 80%. Pollen donors within the forest fragment and group were assigned to 64 and 2 seedlings and 86 and 6 juveniles, respectively. Five of these seedling pollen donors were assigned based on a confidence level of 95% and 61 on a confidence level of 80%; nine of the juvenile pollen donors were assigned based on a confidence level of 95% and 83 on a confidence level of 80%. The average pair-wise coancestry coefficient and its standard error calculated among seedlings and putative mother-trees was 0.249 ± 0.031, and among seedlings and putative father trees it was 0.271 ± 0.035. For juveniles, these estimates were, respectively, 0.258 ± 0.020 and 0.254 ± 0.025. The 95% confidence standard error suggests that all these coancestry values are significantly different from zero.

Table 1 Characteristics of eight microsatellite loci from *Araucaria angustifolia* in the analyzed forest fragment and tree group

Locus	n	k	$\hat{H}_o$	$\hat{H}_e$	Pr(Ex_1)	Pr(Ex_1)
CRCAc2	598	16	0.635	0.676	0.286	0.473
Ag23	600	13	0.758	0.863	0.565	0.724
Ag62	612	14	0.667	0.692	0.321	0.513
Ag45	617	4	0.332	0.417	0.087	0.200
CRCAc1	616	5	0.047	0.062	0.002	0.031
Ag20	610	8	0.546	0.628	0.231	0.411
Ag56	612	8	0.701	0.749	0.347	0.524
As90	602	9	0.405	0.682	0.284	0.468
Mean		9.6	0.511	0.596	—	—
Total		77	—	—	0.931	0.992

H_e, expected heterozygosity; H_o, observed heterozygosity; k, number of detected alleles per locus; n, sample size; (Pr(Ex_1)), exclusion probability of the first; (Pr(Ex_2)), exclusion probability of second parent.

Distance of pollen movement

Pollen was dispersed within the fragment over relatively short distances (Table 2; Figures 2a and b). The distances of pollen movement measured in the seed cohort ranged from 11 to 287 m (average of 83 m; Table 2). The average

Table 2 Profiles of pollen flow for each seed-tree, seedlings and juveniles

Seed-tree	n	*Pollen flow (proportion)*			$\hat{N}_{ep}$	*Within fragment*		*Between fragment and group*
		Fragment	*Group*	*Outside*		*Distance (m)*	$\hat{A}_{ep}$ *(ha)*	*Distance (m)*
Frag_2	18	17 (0.94)	0 (0.00)	1 (0.06)	14.7	133 ± 64	2.6	0 ± 0
Frag_70	18	12 (0.67)	3 (0.17)	3 (0.17)	16.2	90 ± 64	2.6	2069 ± 60
Frag_108	16	14 (0.88)	1 (0.06)	1(0.06)	11.6	91 ± 57	2.0	1946 ± 0
Frag_112	20	17 (0.85)	3 (0.15)	0 (0.00)	12.5	100 ± 46	1.3	1927 ± 0
Frag_135	20	18 (0.90)	2 (0.10)	0 (0.00)	15.4	79 ± 29	0.5	1953 ± 0
Frag_194	18	16 (0.89)	0 (0.00)	2 (0.11)	11.6	85 ± 71	3.2	0.0 ± 0
Frag_200	20	18 (0.90)	0 (0.00)	2 (0.10)	14.3	91 ± 34	0.7	0.0 ± 0
Frag_210	20	19 (0.95)	0 (0.00)	1 (0.05)	9.1	53 ± 26	0.4	0.0 ± 0
Frag_280	20	19 (0.95)	0 (0.00)	1 (0.05)	9.5	80 ± 44	1.2	0.0 ± 0
Frag_369	20	19 (0.95)	0 (0.00)	1 (0.05)	12.4	76 ± 50	1.6	0.0 ± 0
Frag_383	20	20 (1.00)	0 (0.00)	0 (0.00)	11.8	78 ± 36	0.8	0.0 ± 0
Total	210	189 (0.90)	9 (0.04)	12 (0.06)	12.6 ± 2.3	83 ± 52	1.7	2006 ± 59
Group_519	20	11 (0.55)	9 (0.45)	0 (0.0)	7.7	25 ± 23	0.3	1913 ± 75
Seedlings	66	64 (0.97)	2 (0.03)	0 (0.0)	—	70 ± 39	1.0	1979 ± 64
Juveniles	92	86 (0.93)	6 (0.07)	0 (0.0)	—	75 ± 43	1.2	1792 ± 43

n, sample size; pollen flow from within fragment, tree group or outside of the fragment and tree group; effective number of pollen donors ($\hat{N}_{ep}$); mean pollination distance within the fragment and between fragment and group (mean ± s.d.); breeding neighborhood area ($\hat{A}_{ep}$).

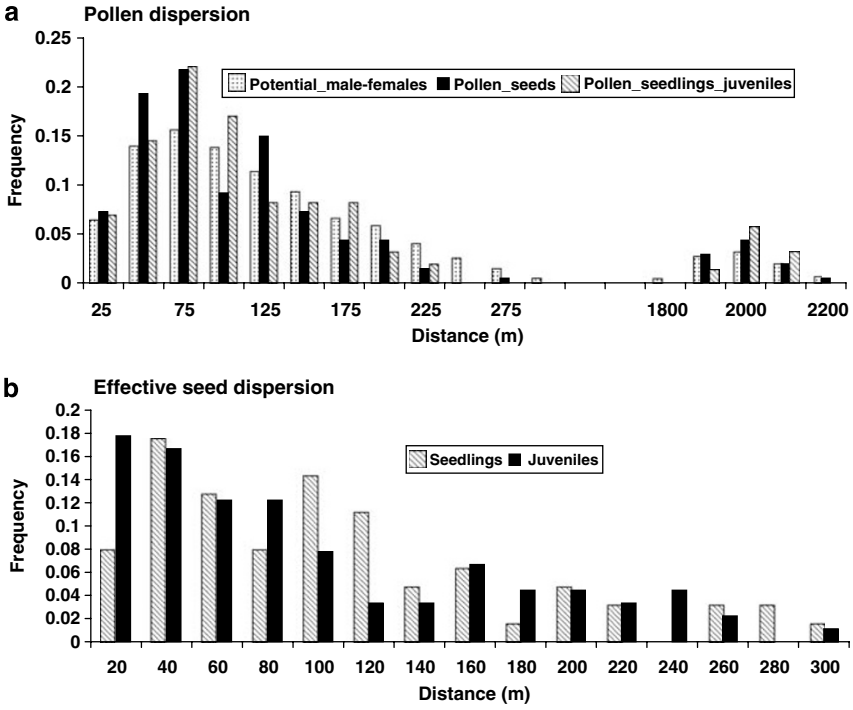

Figure 2 Frequency distribution of potential males and females, pollen-dispersal distances estimated from seeds, and from seedlings and juveniles (**a**), and seed-dispersal distances estimated from seedlings and juveniles (**b**) based on paternity analysis in a small forest fragment and tree group of *A. angustifolia*.

distances of pollen movement between mother and father assignments for the seedlings and juveniles were 70 and 75 m, respectively. About 75% of the seed pollen and 80% of seedling and juvenile pollen traveled less than 150 m (Figure 2a). However, if the group was included in the analysis, comparatively high distances were detected. Mean pollen dispersal distances measured in seeds, seedlings and juveniles for pollen originating from the fragment were 2006, 1979 and 1792 m, respectively, and for the seed cohort in the group the distance was 1913 m (Table 2).

There was a significant negative correlation between the number of seeds ($R^2 = 0.866$, $P < 0.01$), and seedlings and juveniles together ($R^2 = 0.687$, $P < 0.01$) fertilized by pollen donors and the distance between the paternal and maternal trees in the forest fragment. The comparison between the distribution of the potential and effective male parents as a function of the distance to the seed-trees depicts the departure from random mating (Figure 2a). The difference between the distance of pollen donors and all males in relation to the seed-trees (Figure 2a), as evaluated by the Kolmogorov–Smirnov test, was significant in seeds ($D = 0.12$, $P < 0.05$) and in seedlings and juveniles together ($D = 0.11$, $P < 0.05$), indicating that the distances between potential male parents and seed-trees cannot explain the observed mating patterns.

Distance of realized gene flow

The analysis of seedlings and juveniles showed short seed-dispersal distances (Figure 2b). The mothers of all analyzed seedlings and juveniles were found within the fragment, suggesting an absence of seed immigration. Seed dispersal distances for 66 seedlings and 92 juveniles in relation to their putative mothers ranged from 0.35 to 291 m (average of 92 m). About 38% of the seedlings and 47% of the juveniles grew within a 60 m radius of the mother-tree and 60 and 67%, respectively, grew within 100 m; 83% of the seedlings and 80% of the juveniles grew within 160 m (Figure 2a). A significantly high negative correlation was observed between the number of seedlings ($R^2 = 0.5172$, $P < 0.01$) and juveniles ($R^2 = 0.898$, $P < 0.01$) and the distances between mother-trees, seedlings and juveniles.

Effective neighborhood pollination area

On the basis of the seeds, the estimated effective neighborhood pollination area (A_{ep}) within the fragment ranged from 0.4 to 3.2 ha (average of 1.7 ha, Table 2). In the group, this area was 0.3 ha (Table 2). When A_{ep} was estimated based on the seedlings and juveniles, it was quantified to be 1.0 and 1.2 ha, respectively. These areas correspond to a radius around the seed-trees ranging from 31 to 101 m (average of 74 m).

Fixation indices and effective population size in the reproductive population

The fixation indices estimated for adults, male trees, female trees, seeds, seedlings and juveniles from the fragment (Table 3) were positive and significantly different from zero, suggesting deviations from Hardy–Weinberg equilibrium in the population. In the adult population (males and females) the fixation index was higher than in seeds. The highest fixation index was observed in seedlings ($\hat{F}_s = 0.197$). Coancestry coefficients among females, males and males and females together were 0.00023, 0.00103 and 0.00034, respectively (total = 0.00161). This indicates that, on random mating,

Table 3 Parameters estimated for inbreeding and mating systems in *A. angustifolia*

Parameter	*Estimation*
Fixation index in adult generation: F_p	0.150 (0.064–0.278)*
Fixation index in adult female trees: F_f	0.115 (0.007–0.244)*
Fixation index in adult male trees: F_m	0.176 (0.085–0.310)*
Fixation index in seeds: F_o	0.082 (0.023–0.164)*
Fixation index in seedlings: F_s	0.197 (0.097–0.320)*
Fixation index in juveniles: F_j	0.124 (0.015–0.294)*
Paternity correlation: $\hat{r}_p = 1/\hat{N}_{ep}$	0.082 (0.072–0.091)
Coancestry coefficient within families: $\hat{\Theta}_{xy} = 0.125(1+\hat{F}_p)(1+\hat{r}_p)$	0.155 (0.145–0.171)
Variance effective size: $\hat{N}_{e(v)} = 0.5/\hat{\Theta}_{xy}$	3.22 (2.92–3.45)

( ) 95% confidence interval.
*$P<0.05$.

expected biparental inbreeding is very low (0.00034). The estimated effective population size of the reproductive population indicated that 228 adult trees corresponded with 121 unrelated and inbreeding trees ($N_e/N = 0.53$).

The number of pollen donors (N_{ep}) in the fragment ranged from 9.5 to 16.2, with an average of 12.6 per seed-tree (Table 2). From N_{ep}, the paternity correlation (r_p) was estimated to be 0.082, indicating some correlated matings (Table 3). The coancestry coefficient (Θ_{xy}) and variance effective size ($N_{e(v)}$) within progeny were higher than expected in half-sibs (0.125 and 4, respectively).

Spatial genetic structure and estimation of gene dispersal

We found a clear, fine-scale genetic structure within the fragment, indicated by significant positive coancestry coefficients between adult trees, seedlings and juveniles within the distance classes from 0 to 50 m (Figures 3a–c). The average coancestry coefficients for all pairwise adult trees, seedlings and juvenile comparisons, within the first distance class (0–25 m) were estimated to be 0.151, 0.202 and 0.129, respectively. These values are statistically different from zero, according to the 95% confidence interval (Figures 3a–c). Coancestry coefficient values in the distance classes over 50 m decreased to near 0.

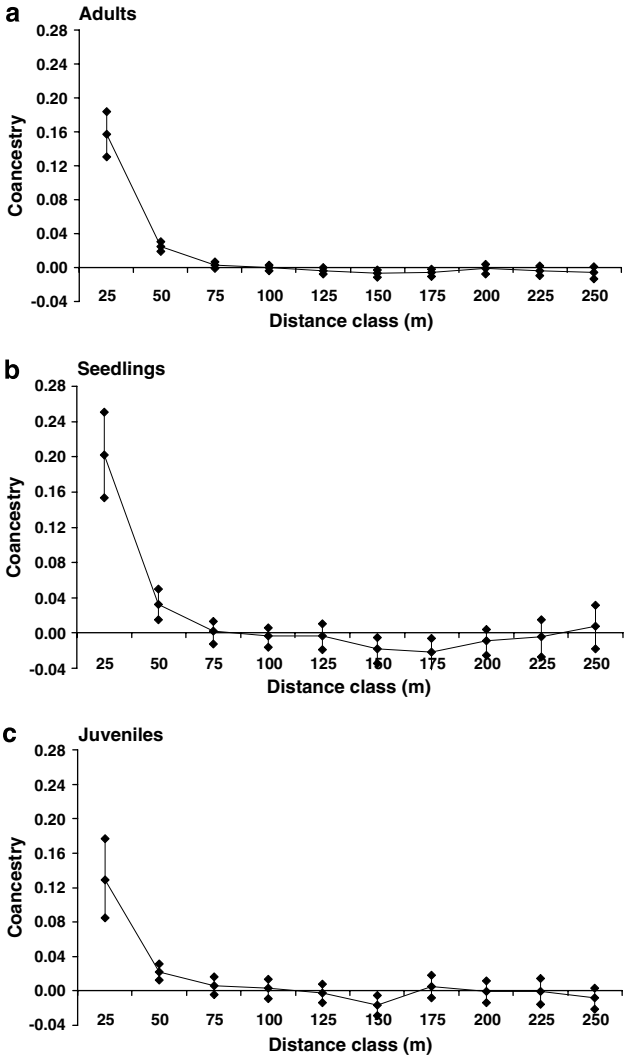

Figure 3 Graphs of coancestry coefficients (θ_{xy}) in 10 distance classes among adult trees (**a**), seedlings (**b**) and juveniles (**c**) within the *A. angustifolia forest* fragment (The vertical lines represent the 95% confidence interval and the solid horizontal line represents the mean coancestry coefficient). *$P>0.05$.

Discussion

Fragment isolation

High levels of pollen flow were observed within the forest fragment, with low levels originating outside the fragment. Paternity analysis of the seeds indicated that 10% of the pollen came from male trees located outside the fragment, of which 4% originated from male trees located in a small tree group about 1.7 km away and 6% appeared to have been produced outside both stands and by trees over 1.7 km away. Similarly, paternity analysis indicated that only 3% (seedlings) and 7% (juveniles) of the realized pollen flow came from outside the fragment (male trees located in the tree group), and that all mothers were detected inside the fragment, indicating an absence of seed immigration. However, it is possible that our pollen immigration rate was underestimated since some foreign gametes may have multilocus genotypes that can also be generated by local males (cryptic gene flow). The probability of cryptic gene flow was high, at 0.478 (1–0.992;[81] Dow and Ashley, 1996), and the number of seeds that matched unrelated males by chance was estimated to be 90 (189×0.478). This represents 43% (90/210) of the total number of seeds sampled in the forest fragment. Thus, the levels of cryptic gene flow may have biased our pollen flow estimates and the actual pollen gene flow might be higher than 10% (between 10 and 47% (189–90 = 99/210) of pollen immigration). The probability of cryptic gene flow in the tree group was low, at 0.07 (1–0.992[9]), and the number of seeds that matched unrelated males by chance was estimated to be 0.6 (9×0.07). This represents 3% (0.6/20) of the total number of seeds sampled in the forest fragment. Therefore, the levels of cryptic pollen flow would not have biased our results in the tree group.

Despite possible bias in the estimates, with a pollen flow (m) ranging from 3 to 10%, and an effective population size (N_e) in the reproductive population of about 121, we can expect a number of immigrants per generation ($N_e m$) ranging from 3.6 to 12.0 individuals. Consequently, genetic differentiation among the present population and other neighborhood populations can

be expected to range between 0.020 and 0.217 ($\hat{F}_{ST} \approx (4N_e m + 1)^{-1}$; Cockerham and Weir, 1993). These results agree with previous estimates of F_{ST} in *A. angustifolia* populations (ranging from 0.04 to 0.102) that showed low genetic differentiation and high historic gene flow among populations (Auler *et al*., 2002; Sousa *et al*., 2003).

The levels of long-distance pollen flow observed in the investigated *A. angustifolia* forest fragment are similar to those detected in other wind-pollinated tree species where the populations are isolated by more than 1 km (Table 4). For example, in a 15 ha *Pinus flexilis* stand isolated by 5 km from neighboring populations, pollen migration was calculated to be 6.5% (Schuster and Mitton, 2000), and in a 20 ha *Pinus sylvestris* stand isolated by 30 km, pollen immigration was calculated to be 4.3% (Robledo-Arnuncio and Gil, 2005). However, these levels of pollen immigration are inferior to those detected in populations not isolated or located at lower distances from further stands (Table 4). For example, in a 5 ha *Quercus macrocarpa* stand isolated by more than 200 m, the rate of pollen immigration was estimated to be 57% (Dow and Ashley, 1998), and in a 9.12 ha stand of *Pinus densiflora* isolated by more than 100 m it was 31% (Lian *et al*., 2001). The present results suggest that the distance to the nearest stand is an important factor determining the rate of pollen immigration and the levels of isolation between the stands. The linear Spearman's correlation between the pollen immigration rate and isolation distance of the stands in Table 4 is negative and significantly different from zero ($\hat{r} = -0.606$, $P < 0.05$), supporting the intuitive hypothesis that more isolated stands receive less pollen immigration. It is likely that levels of pollen immigration decrease and isolation of stands increases with increasing distance between stands, following a typical isolation-by-distance model (Wright, 1943). Thus, high levels of genetic differentiation can be expected among distant remaining forest fragments of *A. angustifolia*.

Tree group isolation

The level of effective pollen flow into the analyzed tree group based on the study of seeds was high (55%), indicating that these trees are not isolated. Pollen flow occurs in both directions, from forest fragment to group and vice versa. A large amount of successful pollen originated from a small number of adult male trees within the stand (three of the nine males). The male situated nearest (11 m) to the study mother-tree was responsible for 67% (6:9) of the seeds assessed within the group. However, the results also suggest the possibility that there is a limitation to pollen flow, since the group is isolated from other *A. angustifolia* trees. All seeds produced by pollen from trees located outside the group have fathers in the adjacent forest fragment (11 trees), predominantly at the southern border of the fragment. The tree group is located in an intensively used agricultural landscape and the probability of its seeds growing into new trees is practically null. However, the pollen contribution of the group to the fragment is important in increasing the effective size.

Distance of pollen flow

The present population of *A. angustifolia* presents two levels of pollen dispersal, a short-distance component within the fragment and a long-distance component between the fragment and the group (Figures 2a and b). Both short and long pollen dispersal distances have been observed in other wind-pollinated species (see Dow and

Table 4 Estimates of pollen flow into populations of wind-pollinated tree species based on paternity analysis

Species	*Stand size (ha)*	*Isolation (m)*	*Immigration rate (%)*	*Reference*
Relative high levels of geographic isolation				
Araucaria angustifolia	5.4[a]	>1700	10	This study
Picea abies	1.0[b]	>4000	16	Xie and Knowles (1994)
Picea glauca	—	>1000	1.0	Adams and Burczyk (2000)
Pinus flexilis	15[a]	>5000	6.5	Schuster and Mitton (2000)
Pinus sylvestris	20[a]	>5000	4.3	Robledo-Arnuncio and Gil (2005)
Pinus sylvestris	22.9[c]	>2000	48	Harju and Nikkanen (1996)
Mean			14.3	
Relative low levels of geographic isolation				
Cercidiphyllum japonica	20[a]	>500	28	Sato *et al*. (2006)
Fraxinus excelsior	—	>600	46–95	Bacles *et al*. (2005)
Picea abies	13.2[c]	0	70	Pakkanen *et al*. (2000)
Picea abies	0.89[c]	>100	83	Burczyk *et al*. (2004b)
Pinus attenuata	0.04[a]	>11	56	Burczyk *et al*. (1996)
Pinus densiflora	9.12[a]	>100	31	Lian *et al*. (2001)
Pinus taeda	2.0[c]	>200	36	Friedman and Adams (1985)
Pseudotsuga menziesii	2.4[a]	0	27	Adams (1992)
Pseudotsuga menziesii	2[c]	0	49	Adams *et al*. (1997)
Pseudotsuga menziesii	—	>100	70	Smith and Adams (1983)
Quercus macrocarpa	5.0[a]	>200	60	Dow and Ashley (1998)
Quercus petrae	5.8[a]	0	69	Streiff *et al*. (1999)
Quercus robur	5.8[a]	0	65	Streiff *et al*. (1999)
Quercus robur	4.5[c]	>400	70	Buiteveld *et al*. (2001)
Mean			54.3	

[a]Natural population.
[b]Plantation.
[c]Seed orchard.

Ashley, 1998; Streiff *et al*., 1999; Schuster and Mitton, 2000; Lian *et al*., 2001; Bacles *et al*., 2005; Robledo-Arnuncio and Gil, 2005; Valbuena-Carabana *et al*., 2005; Goto *et al*., 2006). Within the fragment, the average pollen dispersal distance was less than 100 m and at least 75% of the pollen was dispersed within a distance of 150 m (Figures 3a–c). This average is higher than the average detected for the wind-pollinated trees *Pinus attenuata* (5.43 m, Burczyk *et al*., 1996), *Quercus robur* (22.1–58.41 m; Streiff *et al*., 1999), *Quercus petraea* (18.41–64.56 m; Streiff *et al*., 1999), *Fagus silvatica* (50 m; Wang and Hattemer, 2001) and *Pinus densiflora* (68 m; Lian *et al*., 2001), and similar to the distance observed in *Pinus sylvestris* (83 m; Robledo-Arnuncio and Gil, 2005), but shorter than that calculated for *Pinus flexilis* (140 m; Schuster and Mitton, 2000) and *Fraxinus excelsior* (328 m; Bacles *et al*., 2005). Thus, compared with other wind-pollinated species, *A. angustifolia* can disperse pollen over relatively long distances within the study stand, despite the fact that it has non-saccate and large pollen grains. However, the study stand is small, favoring pollen movement. Further studies of pollen dispersal within large, continuous fragments are needed to confirm the pollen dispersal distances observed here.

The characteristics of *A. angustifolia* pollen and typical high densities of *Araucaria* forests may cause the high proportion of matings within a distance of 150 m and some of the correlated mating ($\hat{r}_p = 0.082$). *A. angustifolia* pollen has a reduced ability to float in the air, and the typically high densities of *Araucaria* forests can create barriers to pollen movement, limiting the distance of pollen dispersal within the forests (Sousa and Hattemer, 2003). El-Kassaby and Jaquish (1996) noted this trend when comparing populations with different densities in *Larix occidentalis*, observing low and nonsignificant levels of correlated matings (0.001 and 0.024) in low-density populations, and higher and significant levels of correlated matings (0.062 and 0.104) in high-density populations. They suggested that a high tree density limits pollen movement within populations. Sousa *et al*. (2005) detected paternity correlations in 13 *A. angustifolia* populations ranging from 0.110 to 0.602. The highest paternity correlation was observed in a 37-year-old *A. angustifolia* plantation with a high density (294 trees/ha), indicating that population density may affect the rate of correlated mating in the species. Dyer and Sork (2001) also observed restricted pollen movement within a *Pinus echinata* continuous forest and reported a negative association between the distance of pollen dispersal and total tree density. A similar situation is likely to exist with *A. angustifolia*.

Another factor that can produce correlated mating in *A. angustifolia* is the variation in individual fertility. Mantovani *et al*. (2006a) studied the reproductive phenology in a population in Campos do Jordγo, Sγo Paulo State during two consecutive years and observed that pollen dispersal and female fertility extend from August to September. Male and female flowering seem to be synchronous, but male flowering phenology showed individual variations in timing and intensity, and some trees tended to produce more strobilos than others, indicating that some males yield more pollen than others (Mantovani *et al*., 2006a). A high proportion of pollen from a small number of individuals could produce correlated matings.

Limitations to pollen dispersal within *Araucaria* forests can produce genetic heterogeneities in pollen gene frequencies among female reproductive trees by correlated matings within small neighborhood pollination areas, increasing the coancestry coefficients within progenies and reducing the variance effective population size. We observed a small average effective pollination neighborhood (1.7 ha) with a coancestry coefficient within families that was higher than expected in half-sib families ($\Theta_{xy} = 0.155$) and a low variance in effective size within families ($\hat{N}_{e(v)} = 3.22$). The calculated A_{ep} is 3.2 times smaller than the area of forest fragment and represents the area where we can expect to find 63% of the pollen parents of a central seed-tree.

The second level of pollen dispersal occurs between the fragment and the tree group, indicating a long pollen-dispersal distance. This result contradicts the hypothesis that pollen flow distance in *A. angustifolia* is low. The long distance of effective pollen dispersal can be explained by high wind intensities and the absence of physical barriers between the fragment and the tree group. Considering these results, we can expect similar levels of pollen flow among populations less than 2 km apart.

Number of pollen donors

Our paternity analysis revealed a large number of pollen donors mating with each seed-tree ($\hat{N}_{ep} = 12.6$; Table 2), indicating that wind is an efficient pollen dispersal vector. However, considering that each *A. angustifolia* cone contains about 200 seeds (Carvalho, 2003) and that there are only 124 reproductive males in the study population, all of the cones will represent a mixture of half-sibs and full-sibs, and the variance in effective population size will be less than the maximum expected in a panmictic population ($\hat{N}_{e(v)} < 0.5/\Theta_{half\text{-}sibs} = 0.5/0.125 = 4$), even with random mating. In addition, the detected spatial genetic structure in the fragment will probably lead to some matings between relatives and to biparental inbreeding. The estimated difference between multilocus and single-locus outcrossing rates in the present data suggests the occurrence of an expressive rate of matings among relatives ($\hat{t}_m - \hat{t}_s = 0.172 \pm 0.018$, $P < 0.01$, analysis not shown), explaining the observed positive and significant fixation indices in the seeds, seedlings and juveniles (Table 3).

Realized gene flow

Our results confirm the hypothesis that seeds in *A. angustifolia* are generally dispersed near the seed-trees. The analysis of fine-scale genetic structure within the fragment detected spatial genetic structure for adult trees, seedlings and juveniles in the distance classes up to 50 m (Figures 3a–c). The average coancestry coefficients were between those expected for half-sibs (0.125) and full-sibs or parent-sibs (0.25). Accordingly, the paternity analysis detected mother-trees of all seedlings and juveniles within the forest fragment. The average distance between seedlings, juveniles and putative mother-trees is approximately 92 m and about 60% of the seedlings and 67% of the juveniles grow within a radius of 100 m of the mother-trees (Figure 2b). In addition, the estimated average value of pair-wise coancestry coefficients among seedlings, juveniles and

mother-trees was near that expected for parent-seed relationships (0.25), confirming the results obtained in the paternity analysis. Spatial genetic structure has also been detected in other stands of *A. angustifolia* (Mantovani *et al*., 2006b). Spatial genetic structure in current *A. angustifolia* forests was expected, considering the high population density (42.2 adult trees/ha), the largeness of the seeds and the fact that seed dispersal in this species occurs primarily by autochory, although the higher density of female trees in the population (19.26 female trees/ha) could have reduced the amount of spatial structure within the stand, owing to the overlap of seed shadows. However, not all females produce seeds every year (Mantovani *et al*., 2006a), and the overlap of seed shadows may not be great enough to reduce the levels of spatial genetic structure. This, combined with the high rate of short-distance pollen dispersal, explains the observed biparental inbreeding in the population, as discussed in a previous section.

Fixation indices

The results showed that there was significant biparental inbreeding in all ontogenic study stages and that the population was not in Hardy–Weinberg equilibrium. However, the fixation index detected in sampled seeds was lower than that detected in adults, suggesting selection against heterozygote individuals. This contradicts the pattern observed in many other tree species, where higher fixation indices have been detected in progenies than in adult trees, suggesting selection against inbred individuals between the seed and adult phases. The high fixation index in adult trees might be attributable to the Wahlund effect rather than biparental inbreeding *per se*, considering the fact that the population is genetically structured. Thus, we used the classical relation among *F*-statistics, $(1-F_{IT})=(1-F_{IS})(1-F_{ST})$ (Wright, 1965) to derive the part of the fixation index in adult trees that originates exclusively in the mating system (biparental inbreeding). In Wrightś expression, F_{IT} represents the fixation index of an individual relative to the total population, and can be attributed to a combination of mating system (biparental inbreeding in dioecious species) and Wahlund effect. In this sense, $F_{IT}=F_p$ since F_p was calculated using all individuals in the population (same and different subpopulations), F_{IS} is the fixation index of an individual relative to the subpopulation and this statistic is affected only by the mating system. The F_{ST} statistic corresponds to the coancestry coefficient among individuals within subpopulations (attributed to genetic drift—the Wahlund effect). In this way, we can assume that $F_{ST}=\theta_{xy}$ and that the present adult population is subdivided into many circular subpopulations within a radius of 25 m, where the higher values of coancestry coefficients from spatial genetic analyses were observed. The fixation index originating in the mating system was estimated as $\hat{F}_{IS}=1-((1-\hat{F}_p)/(1-\hat{\theta}_{xy}))$. The use of the last expression gave another picture of the genetic structure of the population. We conclude that in the adult population there is no biparental inbreeding ($F_{IS}=-0.008$), and that the previous positive F_p value probably reflects the spatial genetic structure of the population. A comparison of this value with the fixation index estimated in the seeds suggests that, in the study population, the strongest selection against inbreeding occurs between the seed and adult phases (inbreeding depression), in agreement with what has been observed in many other tree species.

Implications for conservation, tree breeding and seed harvesting

These results have important implications for seed collection strategies for *ex situ* conservation of *A. angustifolia*, tree breeding and reforestation. The presence of spatial genetic structure in the fragment indicates that seeds for *ex situ* conservation or tree breeding should be collected from trees at least 100 m apart to reduce relatedness among harvested seed-trees. The inclusion of related families in *ex situ* germplasm and progeny tests reduces the effective population size and results in genetic parameters, such as heritability and genetic gains, being overestimated. However, considering the finite number of males within the fragment, the detected spatial genetic structure within 50 m, and the fact that 75–80% of the effective pollen originates from within a radius of 150 m around the seed-tree, we can expect that seeds will be composed of a mixture of half-sibs and full-sibs and, furthermore, represent some level of biparental inbreeding. Thus, estimates of genetic parameters in progeny tests using seeds from small forest fragments (<10 ha) need to be corrected to accommodate correlated matings and biparental inbreeding. The comparably high detected pollen flow distance suggests that seed orchards need to be geographically isolated by more than 1.7 km, preferentially more than 3 km, from the nearest *Araucaria* stand to avoid pollen contamination. The relatively high number of pollen donors across seed-trees in the fragment indicates that female trees in the isolated groups throughout the landscape are suitable for seed collection. Finally, pollen immigration into the fragment from isolated groups of trees highlights the importance of such groups of trees in the promotion of genetic variation, reduction of coancestry and inbreeding, and increase in effective population size in the fragments.

Acknowledgements

JVB would like to thank the Conselho National of Desenvolvimento Cientvfico e Tecnolσgico (CNPq) for granting her a PhD scholarship at the University of Reading (UK). AMS is supported by a CNPq post-doctoral scholarship at the Institute for Forest Genetics and Forest Plant Breeding, Federal Research Center for Forestry and Forest Products, Germany. The authors thank Jutta Buschbom and Carlos Eduardo Svcole Seoane for discussion and important suggestions for an earlier version of the manuscript. We also thank to Associated Editor Thomas R Meagher and two anonymous referees for important suggestions and constructive criticism of the previous article, and Judy McBride for final corrections to the English.

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