Fine-scale genetic structure and gene flow in a semi-isolated population of a tropical tree, *Shorea robusta* Gaertn. (Dipterocarpaceae)

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Sal (*Shorea robusta*) is a wind-pollinated, hermaphrodite tropical forest tree species in South Asia that has suffered significant habitat loss. Despite its economical and ecological importance in the region, so far, no information about its population genetics is available. Using four highly variable nuclear microsatellites, we compared the genetic diversity between adults and juveniles in a semi-isolated natural population in Nepal, quantified its micro-spatial genetic structure by spatial autocorrelation and assessed its pollen and seed flow. Results showed that genetic diversity (H_E = 0.68, N_A = 11) was within the range found in other members of the genus. Spatial aggregation of related individuals was weak both in juveniles and adults trees. Selfing rate was 6%, and mean pollen and seed dispersal distances were 194 m and 35 m respectively. The effective neighbourhood size was estimated to be 4,153 trees. The spatial genetic structure and gene flow data presented here can serve as guidelines for conservation and sustainable use of genetic resources of this tropical species.

Keywords: Dipterocarpaceae, gene flow, genetic structure, population, *Shorea robusta*.

THROUGHOUT the world there is growing concern about the uncontrolled exploitation of tropical forests causing, in particular, a depletion of forest biodiversity. In many cases, people depend on subsistence farming for their livelihoods, which is accompanied by a significant loss of native forests. In Nepal, approximately 80% of the working population lives in rural areas, strongly affecting forest regions. Total deforestation rate was approximately 1.4% per year between 2000 and 2005, indicating that this country lost an average of 530 sq. km of forest annually[1].

Certain species such as sal (*Shorea robusta* Gaertn.) suffer especially from over-exploitation and illegal logging[2]. Although the Government of Nepal declared sal as an endangered tree species, due to lack of law enforcement its loss is continuing unabated. Therefore, detrimental effects on the gene pool, and thus to the fitness of this species, are likely. Findings of its genetic diversity, non-random spatial genetic structure (SGS) and its gene dispersal through pollen and seeds would support its conservation and sustainable use; however, these data are virtually non-existent.

Genetic diversity may vary among populations, major parts thereof or even smaller groups such as cohorts within demes potentially be affected by environmental factors (including anthropogenic influence), life history and demography[3,4]. Long-distance seed flow – even when pollen flow is limited – will lead to reduction in a clumping of similar genotypes. On the other hand, when seed dispersal is limited even when pollen flow is random, a spatial aggregation of similar genotypes is to be expected. Consequently, in 10 neotropical tree species, Hardy *et al.*[5] found higher SGS in species with limited seed dispersal, for instance, for barochorous trees or for tree dispersal by scatter-hoarding animals, than in species that disperse their seeds by wind, birds or bats.

In tropical forests, wind pollination is rare and, hence, few scientific studies are available. For instance, wind-mediated dispersal of pollen in the tropical pioneer tree, *Cecropia obtusifolia*, extended up to 10 km (ref. 6). Most studies on tropical trees have focused on animal-pollinated species (see Table 1)[7]. In insect-pollinated Dipterocarpaceae, the effective pollen dispersal was estimated to be up to 1000 m (ref. 8), whereas data for wind-pollinated members of this taxon are not available.

Sal (*Shorea robusta* Gaertn), a member of Dipterocarpaceae, is one of the dominant tree species of the dry-deciduous tropical forest of South Asia. It is a moderate-to-slow-growing, hermaphrodite species which attains a height up to 35 m and a diameter at breast height (dbh) between 0.60 and 1.30 m in approximately 100 years. The species is naturally found in Bhutan, Bangladesh, India and Nepal, and covers more than 12 million hectares[9]. In Nepal, it is mainly found in the southern part of the country forming an east to west transect. Along river valleys it penetrates deeply into the midlands and along the lower slopes of hills, sometimes up to 80 km from the plains. Single individuals can survive at altitudes up to 1500 amsl, but the species is not commonly found above 1000 m.

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Table 1. Results of spatial genetic structure (SGS) analysis in a natural population of *Shorea robusta*. Kinship coefficient (F_{ij})[21], the coefficient of variation of the number of times each individual is represented (CV partic), the proportion (%) of all individuals represented at least once in the interval (% partic)

Distance class (m)	10	20	30	40	50	60	70	80	90	100
Adults										
Number of pairs	280	670	894	880	799	711	509	400	189	88
% Partic	91.4	100	100	100	100	100	100	98.1	78.1	54.3
CV Partic	0.71	0.62	0.45	0.35	0.38	0.46	0.61	0.83	1.16	1.75
F_{ij}	0.013	0.004	0.005	0.003	0.002	–0.015	0.008	–0.008	–0.008	0.028
Upper confidence interval (95%)	0.017	0.009	0.008	0.009	0.009	0.009	0.012	0.013	0.019	0.029
Lower confidence interval (95%)	–0.017	–0.011	–0.009	–0.009	–0.010	–0.011	–0.012	–0.014	–0.021	–0.030
Juveniles										
Number of pairs	244	679	810	719	554	355	234	92	50	
% Partic	94.3	100	100	100	100	96.6	78.2	40.2	23	
CV Partic	0.61	0.48	0.33	0.28	0.34	0.59	0.92	1.54	2.26	
F_{ij}	0.022	0.005	–0.002	0.007	–0.001	–0.006	–0.001	–0.031	0.010	
Upper confidence interval (95%)	0.016	0.009	0.008	0.009	0.010	0.012	0.016	0.025	0.040	
Lower confidence interval (95%)	–0.014	–0.008	–0.007	–0.008	–0.009	–0.012	–0.015	–0.031	–0.038	

Unlike other *Shorea* species, which are insect-pollinated[10], sal pollen is transported by wind[11]. Expectedly it lacks nectar in flowers, has aerodynamic pollen sizes between 20 and 60 nm, conspicuous drooping inflorescence with pendulous flowers, explosive pollen release caused by moderately gusty winds, and long period of pollen viability (*c*. 50 h)[11]. Seeds of sal are ovoid in shape (approximately 8 mm in diameter), weighing up to 2 g, with two shorter and three longer (up to 75 mm) wings[12]. Seed dispersal is wind-driven (autorotating whirlybirds). Because seeds of *S. robusta* are recalcitrant, viability is lost within a week after falling to the ground[9].

The main objectives of this study are: (i) to characterize the spatial structure in juvenile and adult trees, and (ii) to assess gene flow within a semi-isolated population of *S. robusta*. Although our own studies have reported about the genetic diversity within and between populations of *S. robusta*[13,14], to the best of our knowledge, there have been no studies on SGS and gene flow by means of molecular markers in the species. We used adults and juveniles because the comparison of SGS between the two cohorts can provide insights into the mechanism underlying SGS within populations at the different life stages of *S. robusta*. Moreover, comparing the genotypes of adults and juveniles provides an opportunity to estimate gene flow within population. We have applied highly variable microsatellites and hope that the present findings will contribute to an effective strategy for the management and conservation of genetic resources of this species.

Materials and methods

Plant material

Sampling was carried out in a pure and isolated natural population of sal (27°23′47.8″N, 85°03′23.5″E, altitude: 460 amsl) consisting of 105 adult trees located close to the city of Hetauda (Figure 1). The nearest neighbouring sal populations are found at approximate distances of 1.1 km south, 1.5 km north, 3 km west and 10 km east of the study site. The populations located north and south of the studied site are several hundred hectares in area, whereas the population in the west is scattered comprising <50 ha. There are no scattered single trees closer to the studied site. Tree density of the population amounted to 59 trees/ha (area = ~ 1.80 ha). From this population, all 108 adult trees and 88 juveniles randomly selected from the natural regenerations were sampled (Figure 2). In some part of the population juveniles were absent or found in few numbers, probably due to grazing by livestock. Age of these juveniles ranged approximately from 2 to 5 years, as estimated by plant size when sampled.

According to our communications with local people, there are no records of any mortality or logging of adult trees during the last seven years in the population. In order to carry out a DNA analysis, leaf samples of adults and juveniles were collected, marked and stored in silica gel. Samples were stored at –40°C before processing. Geo-coordinates of each individual were recorded using a Geographical Positioning System (Garmin GPS MAP 60CSx). Although part of the data from the adult trees of the population was used in our earlier studies[13,14], because of the different scope of the current study we also included them in the study.

Genotyping

DNA from silica-dried leaves was extracted using the DNeasy 96 Plant Kit (QIAGEN, Hilden). For the analysis of DNA samples, four microsatellites, i.e. Sle 267, Sle 303a, Sle 562 and Sle 566, developed for *Shorea leprosula*[15] and optimized for *S. robusta* were used[13].

Initially we tested 27 published *Shorea* microsatellites in *S. robusta*; however, only four were found reliable and highly polymorphic and were then used for this study. Polymerase chain reaction (PCR) amplification of these microsatellites was performed in 15 µl reaction mixture, which consisted of ~5 ng of template DNA, 50 mM KCl, 20 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM of each dNTP, and 0.5 U of Platinum^R *Taq* DNA polymerase (Invitrogen). Amplification was carried out using the PTC-200 gradient cycler (MJ Research). The PCR conditions were as follows: an initial denaturing step of 3 min at 94°C, 33 cycles of 94°C for 1 min, 52–55°C (52°C for Sle 562; 55°C for Sle 267, Sle 303a and Sle 566) annealing temperature for 30 s, and 72°C for 1 min, followed by 8 min at 72°C for the final extension step. Genotyping of the microsatellite DNA fragments after PCR was carried out using CEQ^{TM} 8000 Genetic Analysis System (Beckmann Coulter, Fullerton, USA). To show the nature of polymorphism of microsatellites used in this study, chromatograms of two primer pairs for adults and juveniles are shown in Figure 3.

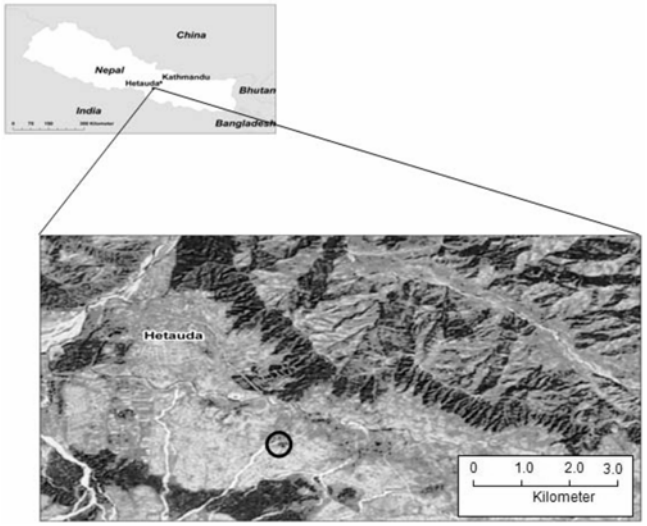

Figure 1. Map of Nepal showing the location of the isolated *Shorea robusta* population (encircled) and a detailed view of its neighbouring forests. Dark pattern indicates other sal forests with the exception of small forest patches located east of the studied population, which are plantations of other forest tree species.

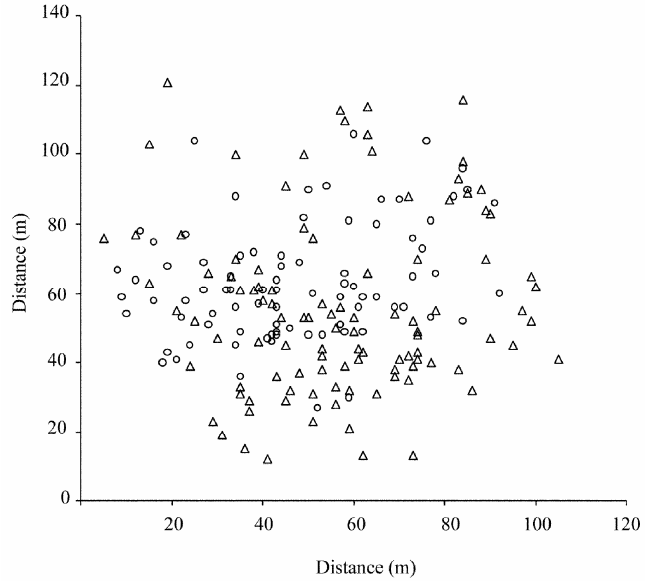

Figure 2. Spatial distribution of adult trees (open triangles) and juveniles (open circles) of *S. robusta* in the Hetauda population.

Statistical analysis

We used the program GENALEX[16] to estimate the following population genetic parameters: observed (H_O) and expected heterozygosities (H_E), number of alleles (N_A), effective number of alleles (N_E) and Wright's F_{IS}. Test for deviation of genotypic frequencies from Hardy–Weinberg expectation and linkage disequilibrium between loci (LD; exact probabilities) was performed using the program GENEPOP[17].

Although several statistical approaches are available to estimate SGS in plant species[3,18], mainly there are two commonly used statistics, i.e. Moran's *I* and kinship coefficient. Moran's *I* coefficient has been widely used, but recently the kinship coefficient is gaining more popularity[19]. Vekemans and Hardy[4] reported that kinship estimator of Ritland[20] tends to give downward biased estimates when rare alleles occur. In contrast, simulation studies have shown that the estimator of Loiselle *et al.*[21] can perform well even for predominantly selfing species with high fixation index[18]. Therefore, we used the kinship coefficient (F_{ij}) of Loiselle *et al.*[21] and the program SPAGEDI[22]. To test the significance of the observed values of F_{ij}, 95% lower and upper confidence intervals were calculated by permutation tests with 10,000 replications. The size of each distance class was set to 10 m in order to facilitate comparisons between juvenile and adult trees.

Our initial intention was to sample open-pollinated seeds from single trees to perform a paternity analysis. Unfortunately, seeds were not available during our sampling trip. Therefore, we had to use the juvenile data to assess gene flow (pollen and seed dispersal distances) within population of *S. robusta*. Assignments of both parental trees (pollen and female gamete contributor) for the juveniles were carried out following the maximum likelihood approach using the program CERVUS[23]. For the simulation analysis genotyping error was set at 1% and the confidence level for LOD (overall log likelihood ratio) at 80% (ref. 24). Since the population is semi-isolated and we detected 11% of pollen coming from outside of the stand, the candidate parents sampled was set at

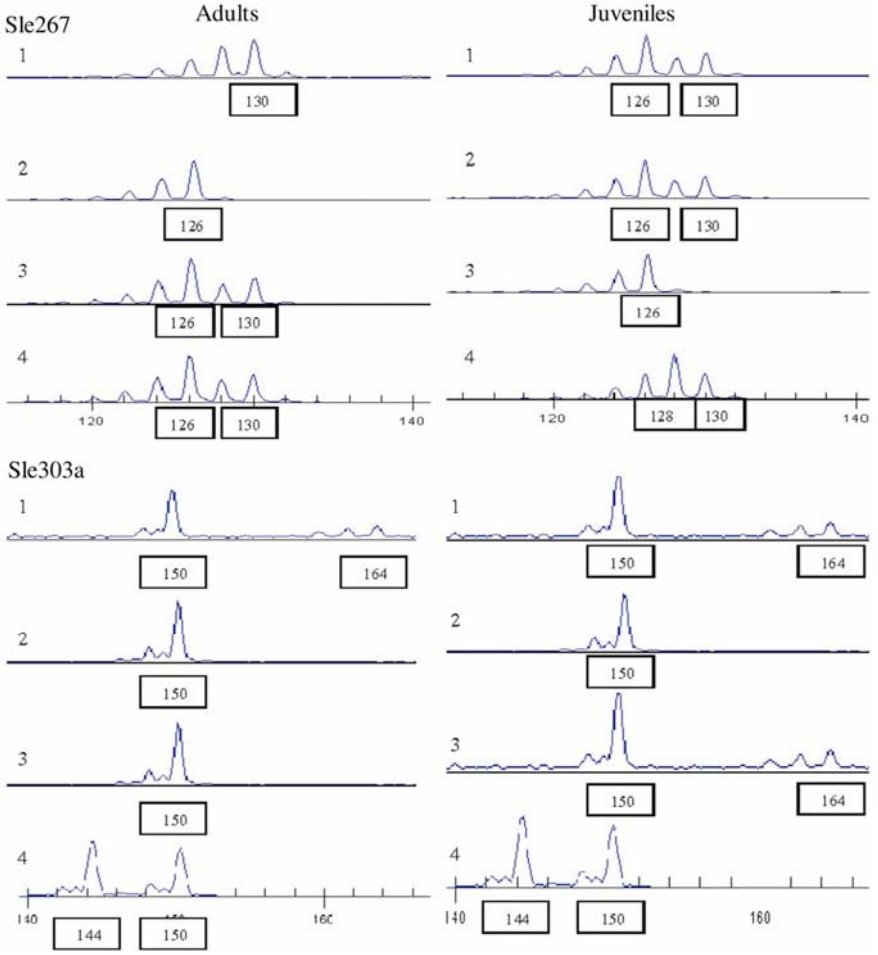

Figure 3. Chromatograms of two microsatellite makers (Sle 267 and Sle 303a) showing polymorphism in four adult and four juvenile individuals. Each juvenile and adult with the same number (1, 2,…) share at least one common allele together. The numbers in the box represent the size (base pairs) of the alleles.

89%. In case the program assigns multiple parental pairs, the pair with highest LOD value was selected. After assigning the most likely combination of parental trees for each juvenile, distances of pollen flow and seed dispersal were measured. For this purpose, the nearest parent was considered as the mother tree and the farthest as pollen donor. This assumption is not free from bias, especially when long-distance seed dispersal by animals is not excluded. However, seed dispersal in *S. robusta* is mainly – if not exclusively – by gravity. The distance between two parents was measured as pollen flow distance, and the distance between the juvenile and the nearest parent was considered as the seed dispersal distance. In the case of pollen coming outside of the stand, the distance between the stand and the nearest sal forest was considered. Average distances for pollen flow and seed dispersal within the population were estimated from these measurements. Pollen dispersal distance of juveniles originated from selfing was considered as 0 m while calculating mean value. Selfing rate was estimated based on the observed number of juveniles that showed a single parent as the source for pollen and seed.

Effective neighbourhood size was estimated according to Crawford's formula[25] modified by Epperson[26]: $N_e = 4\pi D(\sigma_s^2 + \sigma_p^2 (1 - s)/2)$, where D is the tree density, σ_s^2 and σ_p^2 are the dispersal variances of seed and pollen respectively, and s is the selfing rate.

Results and discussion

Genetic diversity

Estimates of genetic diversity in adult trees and juveniles of the Hetauda population are presented in Table 2. The number of alleles per locus ranged from 6 to 19 in adults and 5 to 16 in juveniles. The number of alleles (N_A) for adults and juveniles on average was 11.8 and 10 respectively. Both adults ($N_E = 4.1$) and juveniles ($N_E = 4.0$) had a similar level of an average effective number of alleles. Although expected heterozygosity ($H_E = 0.68$) was equal in adults and juveniles, observed heterozygosity was slightly higher in juveniles ($H_O = 0.70$) than in adults ($H_O = 0.66$). The genetic diversity observed in adults and

Table 2. Genetic diversity of adults and juveniles (natural regeneration) of *S. robusta*: number of samples (N), number of alleles (N_A), effective number of alleles (N_E), observed heterozygosity (H_O), expected heterozygosity (H_E) and fixation index (F_{IS})

Locus	N	N_A	N_E	H_O	H_E	F_{IS}
Adults						
Sle 267	103	11	2.1	0.53	0.53	–0.01
Sle 303a	105	6	2.2	0.55	0.55	–0.01
Sle 562	104	11	4.1	0.79	0.76	–0.04
Sle 566R	105	19	8	0.78	0.88	0.11
Sub-average	104.25	11.8	4.1	0.66	0.68	0.01
Juveniles						
Sle 267	86	5	2.6	0.72	0.62	–0.18*
Sle 303a	88	7	1.9	0.47	0.47	0.01
Sle 562	88	12	4.6	0.80	0.78	–0.03
Sle 566R	86	16	6.9	0.81	0.85	0.05
Sub-average	87	10	4	0.70	0.68	–0.04
Average	95.63	10.9	4.05	0.68	0.68	–0.01

*Significant deviation from Hardy–Weinberg expectation ($P \leq 0.05$).

seedlings in the population studied appears to be similar to other dipterocarps. Our estimates of heterozygosity and average number of alleles ($H_E = 0.68$, $N_A = 10.9$) are within the range also found in *S. leprosula* ($H_E = 0.70$, $N_A = 11.4$)[27], *Shorea curtisii* ($H_E = 0.64$, $N_A = 7.9$)[28], *Dryobalanops aromatica* ($H_E = 0.71$, $N_A = 5.1$)[29] and *Neobalanocarpus heimii* ($H_E = 0.78$, $N_A = 8.8$)[8]. Moreover, it must be taken into account that our estimates were based only on a single, semi-isolated population, whereas in the above-mentioned studies several populations within the natural range were mainly considered. Contrary to our expectation, F_{IS} estimates did not differ between adults and juveniles since in most mixed-mating tree species fixation indices show an excess during early life stages and a deficit of homozygotes later on due to a selection against inbreeds[30]. The lack of different genetic structures in juveniles and adults previously led us to assume a low selfing rate. This assumption was later confirmed by our gene flow estimates.

Juveniles showed slightly higher observed heterozygosity than the adults. This could be due to influx of new alleles in the populations through external pollen, because we found 11% of the juveniles possessing different alleles than the adult individuals. Deviation from Hardy–Weinberg expectation was exclusively significant at locus Sle 267 in the juveniles. However, when the data of both demographic groups were combined, no significant deviation was found (Table 2). No significant linkage between the four loci was found.

Spatial genetic structure

Results of SGS are depicted by the correlograms (Table 1 and Figure 4). Juveniles showed significant deviations from spatial randomness up to a distance of ~ 15 m (10–20 m distance class), whereas no significant SGS was detected for the adults in shorter distance classes. However, a significant negative spatial genetic correlation was estimated at a distance of ~ 60 m (50–60 m distance class).

The results indicate that genotypes are randomly distributed in space across generations. Significant deviations from spatial randomness in single distance classes were detected; however, they were extremely weak. Aggregation of similar genotypes was found up to 15 m distance in the natural regeneration and in the adults in a long-distance class (50–60 m). We are well aware that the sample sizes were relatively small in our study. Although for the adults all available trees were sampled, the number of loci used was limited. Therefore, correlation between real and estimated SGS is probably not high[31]. Thus, the lack of a pronounced SGS should be interpreted that either no SGS in *S. robusta* exists in nature or that a weak SGS was possibly not detected in our study. Despite these limitations, we conclude that a strong SGS does not exist in our studied population. Small differences in SGS during different life stages were also reported in other *Shorea* species. Stronger SGS was detected in juveniles than in adults in *S. leprosula*[27], while also inverse results were found in *S. curtisii* and *Shorea macroptera*[32] as well as in many other tropical trees, such as *Dicorynia guianensis*[33], and *Jacaranda copaia*[34]. Due to the lack of a pronounced SGS in our studied population, one may conclude that an overlap of seed shadow and long-distance gene flow through pollen in *S. robusta* is common, which was supported by our gene-flow data.

Gene flow and effective neighbourhood size

Average exclusion probability for parent pairs of locus Sle 267, Sle 303, Sle 562 and Sle 566R was 0.57, 0.48, 0.73

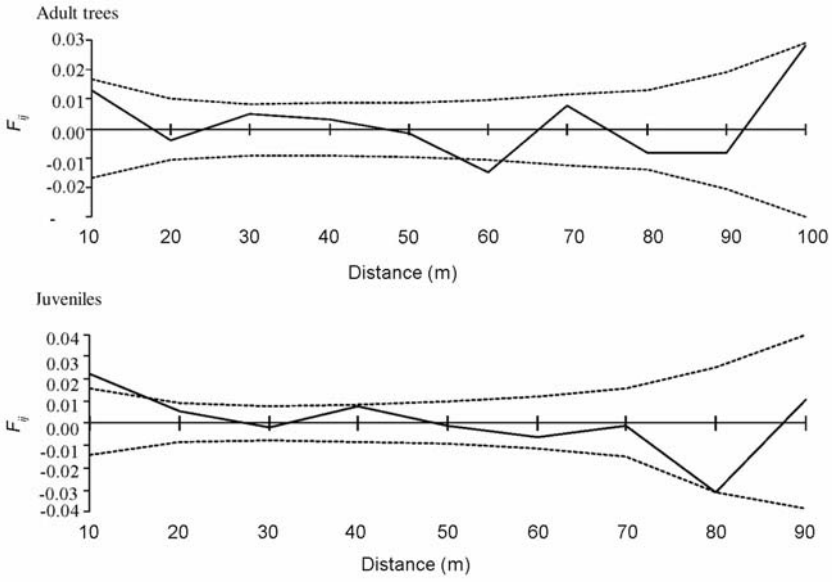

Figure 4. Correlograms of average kinship coefficient (F_{ij}) estimated for adult trees and juveniles of *S. robusta*, and plotted against geographical distance intervals. Dashed lines represent upper and lower 95% confidence limits obtained after 10,000 permutations.

and 0.90 respectively. Paternity assignment for the pair of parents using the maximum likelihood method was possible for 78 juveniles (89%) with both simulations analyses (90% and 100% potential parents sampled).

The analyses of parentage in single populations of tropical tree species have significantly improved our knowledge in this field[8,33,35]. Our study resulted in estimates of mean pollen and seed dispersal distances of 194 (SD = ± 329 m) and 35 m (SD = ± 22 m) respectively. The maximum pollen and seed dispersal distances were 1100 and 105 m respectively. However, the effective long-distance pollen dispersal could not be considered for this estimate of pollen flow. It is well known that long-distance pollen flow can be extensive (see table 2 in Petit and Hampe[36]). In this study, 10 individulas (11%) in the natural regeneration received alleles from outside pollen sources. This could be due to prolonged pollen viability and stigma receptivity (both ca. 50 h)[11], which may facilitate higher success rate in cross-pollination in *S. robusta*. Five (6%) juveniles originated from selfing. Presence of this lower level of selfing in *S. robusta* could be as a result of its cryptic self-incompatibility favouring outcrossing over selfing[11].

Our estimates of seed dispersal in sal ranged from a few to 105 (mean 35 m), and are comparable to other *Shorea* species which all have wind-dispersed seeds[37]. Although we believe that our estimates are the most likely ones, it should be cautioned that gene flow estimates are prone to errors. This must be considered when drawing general conclusions on practical implications. Thus our estimate of the neighbourhood size certainly represents the lower limit, simply because long-distance pollen flow could not be taken into account. However, as a first approximation for this endangered species, we estimated its effective neighbourhood size, approximately 4,153 individuals based on a density of 59 trees/ha.

Conclusion

Keeping the limitations mentioned above in mind, the following conclusions may be drawn for the conservation and sustainable management of the genetic resources of this species. SGS was weak within the single population of our study. Since genetically related juveniles are spatially aggregated in short-distance classes, a thinning at an early stage would already decrease SGS. Moreover, sal seed collection does not require special attention of SGS. As adult trees did not genetically aggregate at short distances, collection of seeds from randomly distributed individuals would be genetically representative of the population. However, a minimum distance between two seed trees should exceed 35 m, which is the mean distance of seed dispersal and would probably avoid sampling of seeders within the family structure. This guideline may also be helpful for the collection of planting material for provenance trials. If sal populations are to be kept genetically isolated, for instance, for *in situ* and *ex situ* conservation, as well as breeding populations for tree improvement, the maximum distance of effective pollen flow must be considered.

The recent development and validation of mechanistic wind dispersal models for airborne pollen and seeds has shown that analytical dispersal models based on average environmental conditions are only sufficient for determining dispersal close to the source[38]. Since wind dispersal of pollen may be best described by Lagrangian models that also account for rare extreme airflows, and we found more than 10% of genes translocated from one or more

sources at least 1,100 m away, we would like to suggest that isolation zones for *S. robusta* conservation units should exceed several kilometres.

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