Gene flow and mating system of the tropical tree *Sextonia rubra*

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Abstract

In this paper we report a study of the mating system and gene flow of *Sextonia rubra*, a hermaphroditic, insect pollinated tropical tree species with a geographic distribution in the Guyana Plateau and the Amazon. Using five microsatellites we analysed 428 seeds of 27 open pollinated families at the experimental site "Paracou" in French Guiana. We observed, compared to other tropical tree species, a high level of genetic diversity. We estimated parameters of the mating system and gene flow by using the mixed mating model and the TwoGener approach. The estimated multilocus outcrossing rate, t_m, was 0.992 indicating nearly complete outcrossing. A significant level of biparental inbreeding and a small proportion of full-sibs were estimated for the 27 seed arrays. The differentiation of allelic frequencies among the pollen pools was $\Phi_{FT} = 0.061$. We estimated mean pollen dispersal distances between 65 m and 89 m according to the dispersal models used. The joint estimation of pollen dispersal and density of reproductive trees gave an effective density estimate of 2.1–2.2 trees/ha.

Key words: Genetic diversity, gene flow, heterozygosity, microsatellites, mixed mating, tropical tree, twogener.

Introduction

For sustainable management of tropical forests and the conservation of genetic diversity it is important to know the potential for pollen dispersal of the tree species (Kanashiro *et al.,* 2002). Species with broader pollen dispersal are assumed to be less sensitive to the negative impact of logging and forest fragmentation. Recently, highly variable microsatellites have been employed to study the gene flow and mating system of tropical trees (Collevatti *et al.,* 2001; Degen *et al.,* 2004; Dick, 2001; White *et al.,* 2002). These studies showed in most cases unexpectedly long pollen flow distances and the impact of pollinator community on pollen dispersal. Often the tree species being studied occurred naturally at low density.

Kenta *et al.* (2004) measured, even for the relative abundant (> 3 mature trees per ha), insect pollinated tropical tree *Dipterocarpus tempehes* a mean pollen dispersal of 222 m. The mating system and gene dispersal of tropical trees have been proven to be highly dynamic and sensitive to ecological factors (Franceschinelli and Bawa, 2000). There is evidence for a negative relationship between the level of selfing and the density of reproductive trees (Murawski and Hamrick, 1991). The level of outcrossing and the distribution of effective pollen might change from one population to another and from one flowering event to another simply as a function of demographic structure, phenology and pollinator composition and abundance (Kenta *et al.,* 2004; Sork *et al.,* 2005).

The evolution of tropical trees species seems to have favoured characteristics in the reproductive system, such as specialized animal pollination, that allow them to persist at low densities. Hence tropical rainforest trees might, to some extent, tolerate an additional local reduction of their density due to forest exploitation. It is further possible that typically abundant species may be more sensitive to reduction in densities than rare species because they are less adapted to this situation.

Sextonia rubra (Lauraceae) is a hermaphrodite species with a geographic distribution in the Guyana Plateau and the Amazon (Van der Werff, 1997). It produces small, scented white flowers in August and September in French Guiana. Despite the fact that the actual pollinators of *S. rubra* are unknown, the flower attributes suggest that pollination is mediated by small diverse insects (Bawa *et al.,* 1985). Flowers were produced on most large individuals in the population in year 2002 (Cloutier and Hardy, unpublished data). The fruits of *S. rubra* contain a single seed and are produced in January in French Guiana. A genetic structure study made on the adult trees at Paracou has reveal only very weak spatial structure suggesting that the seed have the potential to be dispersed on large distances (Hardy *et al.,* 2005; Vekemans and Hardy, 2004). It has been reported that the seed can be dispersed by birds (Sabatier, 1983). However a significant part of the seeds just falls under the trees by gravity allowing to sample seeds underneath them. *S. rubra* is a canopy tree species that reaches at least 1.50 m d.b.h. at Paracou and 40 m height. It is the third most harvested timber tree species in French Guiana.

In this paper we report a study of the gene flow and mating system of *Sextonia rubra* at the experimental site „Paracou" in French Guiana. This site is characterised by a medium density of this species. Thus our study fills a gap between studies on very abundant tropical tree species like *Symphonia globulifera* with more than 10 individuals per ha (Degen *et al.,* 2004) and studies on very rare species like ficus with less than 0.1 individuals per ha (Nason *et al.,* 1996).

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Material and Methods

Site and Sampling

The experimental trial "Paracou" (5°18'N, 52°53'W) is located in French Guiana near Sinnamary. The forest of Paracou is classified as lowland moist forest on ferralitic soil. The mean annual rainfall in this area is 3,076 mm, with peaks in April–June and December. There are two dry seasons; a long one from August to November and a shorter one called "little summer" in March. The experimental trial consists of 16 plots: 15 are 6.25 ha in size and one covers an area of 25 ha. Since 1984, all trees with a d.b.h. (10 cm have been measured by CIRAD Forêt. On the total surface area of 118.7 ha, 286 trees of *Sextonia rubra* with a d.b.h. ≥10 cm have been identified (2.4 trees/ha). The trees have been mapped. Three silvicultural treatments of different intensities were applied to different plots from 1986 to 1988 but some were left as unlogged controls. 15 years later, the effect of the treatments on the density on *S. rubra* is still visible. There is a significant lower density of *S. rubra* in the logged plots (1.61 trees/ha) compared to the control plots (3.12 trees/ha). A detailed description of the experimental trial and treatments can be found elsewhere (FORGET *et al.,* 1999; SCHMITT and BARITEAU, 1988).

In 2002 we collected 428 seeds from 27 mother trees (10–20 seed each) distributed all over the experimental

Figure 1. – Distribution of *Sextonia rubra* with d.b.h. ≥ 10 cm in Paracou. We sampled 428 seeds from 27 mother trees (start).

site (see *figure 1*). The mother trees were distributed in two groups, one sampled in a logged plots and one in unlogged plots. The seeds were collected on the ground. We paid attention that the distance among the sampled trees was sufficient large to avoid confusing the progeny of one adult with those of another. All seeds with genotypes that did not share at least one allele at all loci with the putative mother tree were excluded from further data analysis.

DNA extraction

For adult trees, a disc of 150 mg of cambium stored in a conservation buffer made of 70% Ethanol and 0.3% β-Mercaptoethanol was used for DNA extractions. The buffer-preserved discs were flash- frozen in liquid Nitrogen and then ground to a powder using a mortar and pestle. Subsequent DNA extraction followed modifications of the widely used CTAB extraction procedure (DOYLE and DOYLE, 1987) (2X CTAB buffer: 100 mM Trisma base, 1.4 mM NaCl, 20 mM Ethylenediaminetetra-acetic Acid (EDTA), 2% Cetyltrimethylammonium bromide (CTAB), 1–4% Polyvinylpyrrolidone (PVP)-40, 0.2% Ascorbic acid, β-mercaptoethanol 0.3%). After germination of the seeds in the greenhouse total genomic DNA was extracted from seedling roots following the same protocol.

Microsatellites

A microsatellite library enriched with GA, CA, GCT and GAC repeat motifs was constructed from *S. rubra* following the procedure of Edwards as modified according to (BUTCHER *et al.,* 2000), with the addition of a second round of hybridization using the same conditions of the first round. Following membrane enrichment, the DNA was cloned using the Topo-TA cloning Kit (Invitrogen). Sixty six colonies, chosen according to the fragment length, were sequenced in a MegaBace™ DNA Analysis System (Amersham) following the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham) guidelines. Forty colonies contained microsatellite dinucleotide motifs, and 5 trinucleotide motifs. Twenty three pairs of primers flanking microsatellite regions were designed using Primer3 software (ROZEN and SKAKESKY, 2000). They were tested for amplification on a batch of 10 samples.

The PCR cocktail (15.0 µL total) contained 2mM of $MgCl_2$, 300 µM of each dNTP, 0.5 µM of each primer, 1x reaction buffer, 1 unit of *Taq* polymerase (Invitrogen Corporation) and 3 ng of DNA. The PCR was performed on an ABI 9700 thermal cycler using the following protocol: 4 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55 or 57°C, and 30 s at 72°C; ending with 4 min at 72°C. The amplification products were visualised by electrophoresis with a LIZ-500 size standard (Applied Biosystems Incorporated, ABI) on an ABI 310 automated sequencer.

Primers correctly amplified for 13 loci. Among them, seven loci were polymorphic (*table 1*). Allele sizes were scored using the program Genotyper from Applied Biosystems. The observed segregation within families showed codominant inheritance of the microsatellite fragments.
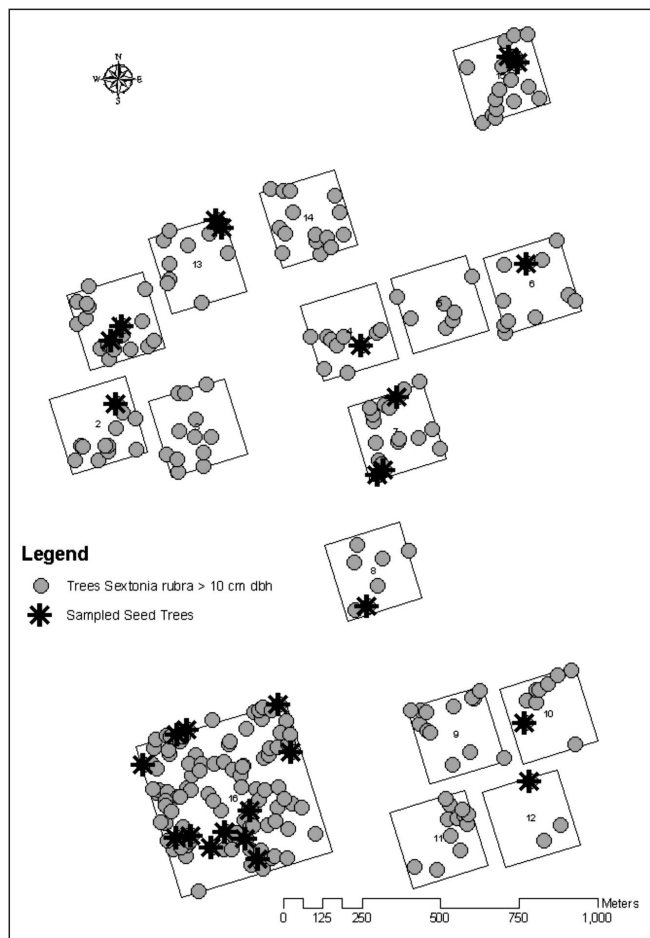
Table 1. – Primer sequences, repeat motif, accession number, annealing temperature, range of fragment length (bp) for *Sextonia rubra*, * = loci used for this study.

Locus		SSRs	Accession number	Annealing temperature	range of fragment length (bp)
Sr3*	F : TGTATGGCACATCTCACAT	$(GA)_{29}$	AJ972417	57°c	167 - 209
	R : AGGTTGGCTAAAAGAAGTG				
Sr9*	F : CACCCCATTGATGAAGGGGC	$(GA)_{18}$	AJ972418	57°c	247 - 277
	R : GCTCACATAGTTGGCAAGAT				
Sr10	F : TTTGCTAATGACCTCTTTC	$(AG)_{22}$	AJ972420	55°c	203 – 233
	R : GAGACACATGCCTTTTT				
Sr 43*	F : ACCATAATGACTAGGCG	$(GA)17$	AJ972421	55°C	117 - 149
	R : TACTCCTCCTCCTTCCTCTT				
Sr 51	F : TTCCTTCCCTTATCACTT	$(TC)_{15}$	AJ972419	55°c	224 - 260
	R : AAAGCACATACATACTCACA				
SrF03*	F: CAATTCAGGGGATGGTCCTT	$(GT)_{15}$	AJ972415	55°c	247 - 265
	R : ACCCAGGAAATACTTCTCAT				
Sr H03*	F: CCCTGACTTCTACTTGCTTC	$(CTT)_{18}$	AJ972416	55°c	249 - 261
	R: ACCCNTACTTTACCTGATGA				

Finally, 5 out of 7 microsatellite loci were chosen for scoring genotypes.

Data analysis

Genetic variation and heterozygosity

For each locus the observed heterozygosity (H_o), expected heterozygosity (H_e), the number of different alleles (A), the effective number of alleles $A_e = 1/(1-H_e)$, and the fixation index $F = 1-(H_o/H_e)$ were calculated for the seeds as described by WEIR (1996). To estimate the significance of F, numerical tests were performed on the basis of Monte Carlo methods (MANLY, 1997). 1000 permutations of homologous alleles among individuals were run to test the significance of the F values for each locus. Each permutation leads to a new random association of the alleles within the sample of seeds and adults (resampling without replacement). After each permutation the F values were recalculated and compared to the observed values. The relative frequency of those cases leading to more extreme F values than the observed values were used to estimate the probability of significant deviation from the Hardy-Weinberg proportions (DEGEN et al., 2004).

Mating system

By use of the mixed mating model (RITLAND and JAIN, 1981) we estimated the single- and multilocus outcrossing rates. The outcrossing rates were calculated with the program MLTR version 2.3 (RITLAND, 2002) by maximum likelihood, fitting the observed proportions of genotypes descended from a known maternal genotype to the proportions expected under the mixed mating model. The model assumes that: (a) no selection and no mutation following fertilization may occur; (b) each mating represents a random event of an outcross or a self-fertilization, with probabilities equal to t and (1-t), respectively (c) there is no assortative mating or variability in pollen pool frequencies (RITLAND and JAIN,

1981). In addition we calculated the correlation of outcrossed paternity within progeny arrays (r_p), which is the probability that a randomly chosen pair of seeds from the same family are full sibs. With version 2.3 of MLTR (RITLAND, 2002) some departures from these assumptions could be accommodated and be treated as additional facets of the mating system. If mating occurs between relatives (biparental inbreeding), the single-locus selfing rate (s_s) should be higher than the multilocus selfing rate (s_m), and the difference provides a minimum estimate of the apparent selfing due to biparental inbreeding. The reason for this expected difference between s_s and s_m is that the more loci used, the lower the likelihood of confusing selfed and biparentally inbred progeny (GRIFFIN and ECKERT, 2003). We performed 500 bootstraps over families to get standard errors for each parameter.

TwoGener

Following SMOUSE et al. (2001) we made a twogener analysis on the progeny arrays of Paracou. The principle of this method is to estimate Φ_{FT}, the differentiation of allelic frequencies among the pollen pools sampled by several mother trees in the population. The relation between Φ_{FT} and dispersal distance has been shown for given dispersal curves (AUSTERLITZ and SMOUSE, 2001), allowing the development of several estimates of pollen dispersal (AUSTERLITZ and SMOUSE, 2002). A general estimate is based on the global Φ_{FT} measured on all the seed trees of the population. This provides an estimate of the pollen dispersal distance (delta) assuming a given dispersal curve and a density of reproducing adults (d) in the landscape. Also the pairwise Φ_{FT} between all mother trees in the population were calculated to design pairwise estimates that allow one to jointly infer the parameters pollen dispersal distance (delta) and a density of reproducing adults (d). As noted by AUSTERLITZ and SMOUSE (2002), the pairwise analysis yields more

accurate estimates of Φ_{FT} but requires larger sample sizes.

We tested the normal and exponential dispersal functions and used, according to former phenological observations, a proportion of the known adult density as entry into the estimation. In Paracou *Sextonia rubra* had a density of 2.28 trees with a d.b.h of at least 10 cm. Among them about 65 % did flower as controlled by phenological observations in 2001. Thus we used a density of 1.5 reproductive trees for the calculations in the Two-Gener approach.

We computed the 99 % confidence interval of Φ_{FT} by bootstrapping among loci with 1000 replicates (WEIR, 1996).

Results

Genetic variation and heterozygosity of the seeds

We measured a mean of 15.6 alleles per locus (A) and an average effective number of alleles (A_e) of 5.82 (*table 2*). For all loci we found a significant excess of homozygotes (mean $F = 0.106$). The loci Sr3, Sr43 and SrF03 had nearly the same F-value of about 0.13. Smaller values were observed for Sr9 and SrH03.

Mating system

The multilocus outcrossing rate (t_m) had a value of 0.992 (0.039). There was a significant difference between the multilocus and single locus estimates t_m-$t_s = 0.137$ (0.043). This implies a visible proportion of

biparental inbreeding in Paracou. We also observed a significant but relative low value for the correlation of outcrossed paternity $r_p = 0.097$ (0.014). Hence, the number of effective pollen donors in a family was relative high. We computed these parameters separately for the 8 families in the logged plot and the 11 families in the unlogged control. But we could not find any significant differences

Gene flow

The distance between sampled seed trees varied between 24 m and 2700 m with a mean of 1165 m (*figure 1*). The differentiation of allelic frequencies among the pollen pools was $\Phi_{FT} = 0.061$ with the 99 % confidence interval from 0.041 to 0.098. The estimates of the mean pollen dispersal (delta) for the normal model were 78.8 m with fixed tree density and 65.9 m for the joint estimation of tree density and delta (*table 3*). The values for the exponential model were respectively 89.0 m and 72.2 m. The joint estimation of delta and density of reproductive trees increased the estimated tree density up to 2.1 trees/ha (normal model) and 2.2 trees/ha (exponential model). The differences for the error, calculated as a quadratic criterion by fitting the observed and expected pairwise Φ_{ij} values (AUSTERLITZ and SMOUSE, 2002), were quite small among the models. The smallest error was obtained for the joint estimation in the normal model. Also the fit to the exponential power function was tested, but the error was larger than for the other models.

Table 2. – Number of alleles (A), effective number of alleles (A_e), observed and expected frequencies of heterozygotes (H_o, H_e), Fixation Index (F) and probability for departure from Hardy-Weinberg-Heterozygosity (P) for seeds of *Sextonia rubra* in Paracou.

Locus	A	A_e	H_o	H_e	F	P
Sr3	23	8.95	0.772	0.888	0.131	0.000
Sr9	15	8.23	0.813	0.879	0.074	0.000
Sr43	19	10.01	0.781	0.900	0.132	0.000
SrF03	12	3.22	0.599	0.690	0.134	0.000
SrH03	9	4.63	0.738	0.784	0.059	0.012
Mean/Gene Pool	**15.6**	**5.82**	**0.740**	**0.828**	**0.106**	**0.000**

Table 3. – Density of reproductive trees and mean pollen dispersal distance (delta) estimated for the normal and exponential dispersal model. The error is a quadratic criterion for the fit between expected and observed values for pairwise Φ_{ij} estimates (AUSTERLITZ and SMOUSE, 2002).

dispersal function	density constraint	density of reproductive trees (N/ha)	delta (m)	error
Normal	fixed	1.5	78.8	0.9081
Normal	estimated	2.1	65.9	0.9071
Exponential	fixed	1.5	89.0	0.9085
Exponential	estimated	2.2	72.2	0.9075

Discussion

Level of diversity

With mean values of $A_e = 5.23$ we observed relative high values for the effective number of alleles at the microsatellites. Most other neotropical tree species had lower genetic diversity. In French Guiana, LATOUCHE-HALLÉ et al. (2003) observed a mean value of $A_e = 3.23$ for the tree species *Dicorynia guianensis*, and in the same region DUTECH et al. (2002) found values between 1.69 and 2.08 for *Voucapoua americana*. A smaller diversity at microsatellites was also reported for *Carapa guianensis* in the Amazon (VINSON et al., 2005). But for *Symphonia globulifera* higher values ($A_e = 8.02$–9.16) were found in Paracou (DEGEN et al., 2004). Comparison among species should be done with caution because of the limited number of analysed loci and possible differences in mutation rate between di- and tri-nucleotide microsatellite loci.

Heterozygosity

For the seeds we observed a significant excess of homozygotes ($F = 0.106$) compared to the expected Hardy-Weinberg proportions. Also for the population of adults a significant excess of homozygotes ($F = 0.076$) was found (HARDY et al., 2005). This excess of homozygotes could be explained by the Wahlund effect, the presence of null alleles, selfing and/or biparental inbreeding. The presence of null alleles and the presence of subpopulations with limited mating contact (Wahlund effect) would result in a stronger variation of the F-values among the loci. But we found a relative homogeneous excess of homozygotes over all loci. The outcrossing was nearly complete ($t_m = 0.992$), thus it is more likely that the excess of homozygotes is caused by biparental inbreeding.

Outcrossing rates

The estimated multilocus outcrossing rates t_m was 0.992. We observed a significant level of biparental inbreeding ($t_m - t_s = 0.137$), which may be explained by limited pollen dispersal among relatives. But we found only a small proportion of full-sibs ($r_p = 0.097$). The small proportion of full-sibs indicates a good mixture of the pollen pool and a good synchronisation of flowering. The presence of bi-parental inbreeding is some how complicated to explain because you would expect a positive spatial autocorrelation for the adults as an indicator of a family structure. But there was nearly no spatial genetic structure (HARDY et al., 2005; VEKEMANS and HARDY, 2004). Maybe a closer look at the flowering phenology will help to understand this observation.

Effective pollen dispersal

By use of the TwoGener approach we estimated for the population in Paracou mean pollen dispersal distances (delta, *table 3*) between 65 m and 89 m. The values differed slightly according to the dispersal model used (normal versus exponential model) and the estimation method (only delta estimation versus joint estimation of delta and density). The joint estimation calculated an effective density of 2.1 reproductive trees/ha for the nor-

mal model and 2.2 for the exponential model. This is probably an overestimation because the measured density of all trees bigger or equal than 10 cm dbh is 2.6 and we know that in most cases only trees bigger than 30 cm dbh participate in flowering. Only in the unlogged plots the overall density is with 3.12 higher than the estimated effective density. Thus the estimated longer distance of pollen dispersal is more reliable.

Using the TwoGener approach SORK et al. (2002) measured a mean pollen dispersal of 64.8 m for the wind pollinated *Quercus lobata* with a density of 1.19 trees/ha. With the same approach DICK et al. (2003) measured a mean pollen dispersal of 1509 m in a fragmented landscape and 212 m in undisturbed forests for the insect pollinated tropical tree *Dinizia excelsa* with a density of 0.3 trees/ha. In comparison to these results the pollen dispersal of *Sextonia rubra* is much shorter. In general pollen dispersal seems to be negatively correlated with the tree density: the high tree density in Paracou led to short pollen dispersal. This was to a more extreme extend reported for *Symphonia globulifera* in Paracou (DEGEN et al., 2004). For this species with densities of 10.9 individuals per ha a mean pollen dispersal of 27 to 53 m was estimated. We compared the estimated pollen dispersal distance in the logged versus the unlogged plots in Paracou. But we did not find any significant difference as has been reported for *Cornus florida*, an animal pollinated species (SORK et al., 2005). One reason for that observation might be the limited number of seed families in the two groups. But at least we can say the differences in pollen dispersal in logged and unlogged plots were not big. So we might conclude that the disturbance at the relative small scale of the logged plots of Paracou (6.25 ha) with surrounding native forest had no impact on pollination distances.

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