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Does long-distance pollen dispersal preclude inbreeding in tropical trees? Fragmentation genetics of *Dysoxylum malabaricum* in an agro-forest landscape

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Abstract

Tropical trees often display long-distance pollen dispersal, even in highly fragmented landscapes. Understanding how patterns of spatial isolation influence pollen dispersal and interact with background patterns of fine-scale spatial genetic structure (FSGS) is critical for evaluating the genetic consequences of habitat fragmentation. In the endangered tropical timber tree *Dysoxylum malabaricum* (Meliaceae), we apply eleven microsatellite markers with paternity and parentage analysis to directly estimate historic gene flow and contemporary pollen dispersal across a large area (216 km^2) in a highly fragmented agro-forest landscape. A comparison of genetic diversity and genetic structure in adult and juvenile life stages indicates an increase in differentiation and FSGS over time. Paternity analysis and parentage analysis demonstrate high genetic connectivity across the landscape by pollen dispersal. A comparison between mother trees in forest patches with low and high densities of adult trees shows that the frequency of short-distance mating increases, as does average kinship among mates in low-density stands. This indicates that there are potentially negative genetic consequences of low population density associated with forest fragmentation. Single isolated trees, in contrast, frequently receive heterogeneous pollen from distances exceeding 5 km. We discuss the processes leading to the observed patterns of pollen dispersal and the implications of this for conservation management of *D. malabaricum* and tropical trees more generally.

Keywords: gene flow, microsatellites, spatial genetic structure, tropical forest restoration, Western Ghats

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Introduction

Loss of continuous forest and habitat fragmentation due to land use change is one of the major causes of global biodiversity loss (Sala *et al.* 2000). Tropical trees increasingly survive only in highly fragmented complex human-dominated landscape mosaic (Myers 1992). Long-distance pollen and seed dispersal are thus critical processes for ensuring reproduction, gene flow and ultimately the persistence of many tree species (Sork & Smouse 2006). Trees are foundation species supporting a substantial proportion of global biodiversity (Ellison *et al.* 2005). Understanding the ecological and genetic consequences of habitat fragmentation for tree species is vital to support science-based biodiversity conservation and ecological restoration.

The genetic consequences of fragmentation in trees, especially regarding gene flow, genetic structure and inbreeding remain a highly debated topic (Kramer *et al.* 2008; Bacles & Jump 2011). Many studies have applied paternity analysis to measure pollen flow, revealing that

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pollen dispersal can occur over long distances, especially in tree species occurring at naturally low densities (Petit & Hampe 2006; Dick *et al.* 2008). Long-distance gene flow is important for maintaining genetic connectivity and thus counteracting genetic drift and inbreeding, which may otherwise undermine population viability (Trakhtenbrot *et al.* 2005). Some tree species have been shown to exhibit enhanced pollen dispersal in fragmented habitats, attributed to the change in the spatial structure as intervening individuals are lost (Young *et al.* 1993; Dick 2001; Kamm *et al.* 2010; Lander *et al.* 2010). Together with long and overlapping generations this has led to the view that tree species in fragmented habitats are rarely genetically isolated (Hamrick 2004; Kramer *et al.* 2008).

There are however numerous studies demonstrating negative genetic consequences of fragmentation in tropical trees, for example, elevated inbreeding (Fuchs *et al.* 2003; Kettle *et al.* 2007; Dick *et al.* 2008; Vranckx *et al.* 2012) and reduced reproductive fitness (Rocha & Aguilar 2001; Cascante *et al.* 2002; Ghazoul 2005; Finger *et al.* 2011). A confounding factor in genetic studies of fragmented tree populations is that adult cohorts often predate habitat fragmentation, which makes it difficult to detect genetic consequences. Empirical studies investigating chronically fragmented temperate tree species (>600 years) show significant reductions of genetic diversity in small patches of mature trees (Jump 2006; Dubreuil *et al.* 2010).

Significant patterns of fine-scale spatial genetic structure (FSGS) occur across a diverse range of tropical tree species (Hardy *et al.* 2006; Harata *et al.* 2011; Kettle *et al.* 2011). One important and under-studied aspect of fragmentation genetics in tropical trees is the interaction between contemporary pollen dispersal and FSGS. Tree species surviving in remnant fragments with strong FSGS may be especially vulnerable to reduced pollen dispersal and consequently elevated biparental inbreeding (Chybicki *et al.* 2011). Additionally, modified pollen dispersal due to fragmentation is likely to change fecundity variance and the effective number of fathers which can exacerbate genetic drift (Klein *et al.* 2008).

To investigate the interaction between contemporary pollen dispersal and patterns of FSGS, it is necessary to evaluate these processes at a spatial scale corresponding to the scales of fragmentation. Only very few studies have evaluated pollen dispersal over ecologically relevant landscape scales, partly because of the logistical challenges this presents of sampling all potential pollen donors over the study area (but see Lander *et al.* 2010; Finger *et al.* 2011, 2012). We apply genotype data at eleven microsatellite markers with paternity and parentage analysis to directly estimate contemporary pollen dispersal at the landscape scale and FSGS in the tropical timber tree *Dysoxylum malabaricum* (Meliaceae). We intensively surveyed an area of 216 km^2 in Kodagu district, part of the Western Ghats biodiversity hotspot in South India (Fig. 1) and sampled all known adult trees of *D. malabaricum*. This exhaustive inventory of adult trees enables us to specifically evaluate the patterns of contemporary pollen dispersal, male fecundity variance, patterns of FSGS and the spatial distribution of adult trees in a complex agro-forest landscape to investigate the effects of land use change on population genetic processes which may underpin population viability.

Our aim in this study is to help resolve the current debate on forest fragmentation genetics by asking the question: does long-distance pollen dispersal mitigate the genetic consequences of habitat fragmentation? Specifically, we apply the following hypotheses as a heuristic framework using *D. malabaricum* as a focal species: (i) fragmentation leads to genetic erosion and increased genetic differentiation; (ii) local density influences patterns of contemporary pollen dispersal and (iii) short-distance pollen dispersal coupled with FSGS leads to elevated

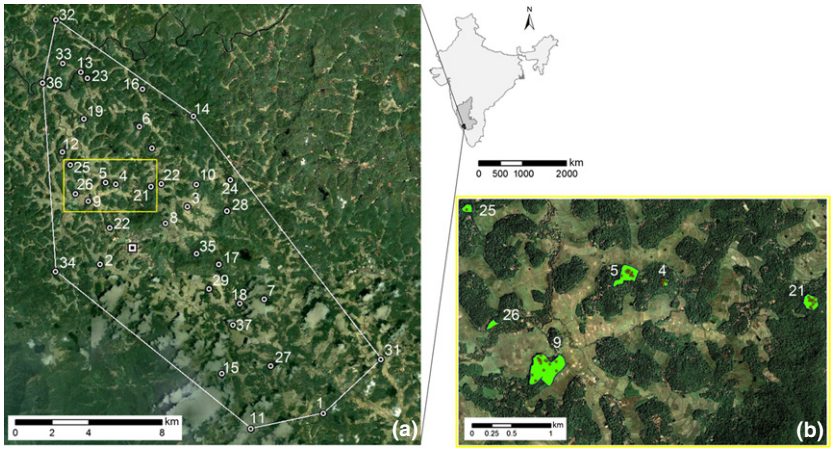

Fig. 1 Location of the study area within India, Karnataka, is displayed in grey. (a) Study area marked as minimum convex polygon around the numbered sampling sites (216 km^2). The small square is the location of Virajpet, the largest City in the area. (b) Zoom of the rectangle on image (a). The polygons in image (b) display sacred groves and the dots within adult *Dysoxylum malabaricum* trees. The number of adult trees, progeny samples and the area per sampling site are provided in Table S1, Supporting information.

inbreeding in highly fragmented landscapes. *Dysoxylum malabaricum* and the sacred grove forests of the Western Ghats provide an excellent study system. Unlike other tropical regions, this region has experienced significant and perpetual fragmentation over several centuries (although this has become more intense in the last few decades) providing a primary example of the types of mosaic landscape (*sensu* Dunning *et al.* 1992) and processes likely to proliferate over much of the low-lying tropics in the coming century. In our study area, *D. malabaricum* is largely confined to sacred groves, having been previously extracted from the remaining forest patches for timber. This study contributes to our understanding of the seemingly contradictory idea that many tree species seem to be robust to forest fragmentation because they maintain high levels of genetic connectivity by pollen dispersal (Bacles & Jump 2011); however, many species show increased biparental inbreeding in fragmented habitats (Vranckx *et al.* 2012). Elucidating this paradox of forest fragmentation genetics is relevant directly to the conservation of *D. malabaricum* and to a plethora of tropical tree species of conservation concern.

Methods

Study site and species

Our study focuses on an area of highly fragmented tropical rain forest in the Kodagu region of the Western Ghats biodiversity hotspot. In the past 100 years, the total area (6275 ha) of sacred grove forest in this region has declined by over 50% to 2549 ha while the number of groves increased from 873 to 1214 (Kalam & Thanuja 2000). This region provides an ideal system to investigate long-term habitat fragmentation that goes beyond habitat loss only. Eighty per cent of the sacred groves are now smaller than two hectares (Kushalappa *et al.* 2001) and contribute only about 2% of the forest cover in the region (Bhagwat *et al.* 2005). The study region is a major coffee-growing region in India (Coffee Board of India 2008 in Garcia *et al.* 2010), where coffee plantations are established under native trees. Expansion of coffee production between 1977 and 2007 resulted in more than 30% loss of primary forest (Garcia *et al.* 2010), where the understory was replaced with coffee and native tree recruitment was prevented by weeding. Further intensification within these coffee agro-forest systems has led to native shade trees being replaced by fast-growing exotic species (e.g. *Grevillea robusta*), further degrading this landscape (Garcia *et al.* 2010). Within our study area, land use includes shade coffee plantations, paddy, sacred grove forests and private forest patches. The study area was chosen to ensure that all trees were a minimum of 2 km from any state-owned continuous forest reserve, so as to reduce the potential for influx of pollen from unsampled pollen donors in continuous forest.

Dysoxylum malabaricum (Meliaceae) is a monoecious tree species with dull greenish yellow hermaphrodite fragrant flowers approximately 7 mm long and 4 mm wide arranged in racemes. Observations of flowering in two consecutive flowering seasons from 2009 to 2010 showed flowering commences in mid-February (with a flowering time of about 4 weeks and peak flowering of about 1 week) and has finished by the end of March. The floral structure and nectar reward suggest insect pollination; small beetles and thrips have been observed on the flowers (S. A. Ismail, personal observation). The fruit contains four carpels with two ovules each, of which normally only one ovule develops. When mature, the fruit splits along the septa into four sections, displaying the seeds (*c.* 30 × 20 mm), which are food for large-gape birds (Ganesh and Davidar 2001) including the Malabar Grey hornbills (*Ocyceros griseus*). Birds consume the seeds one at a time for the brown lipid-rich seed coat, the seed being subsequently regurgitated. If mature fruits drop to the ground with their seed coat still attached, they rot quickly on the forest floor, suggesting obligate zoochory (S. A. Ismail, personal observation).

Dysoxylum malabaricum is a large (reaching heights in excess of 45 m) ecologically and economically important canopy emergent tree, endemic to the Western Ghats. It is extensively logged for its valuable timber (round wood logs of *D. malabaricum* fetched up to 620 US $ per cubic metre at a timber auctions in Kodagu (S. A. Ismail, personal observation 2008). The species is not catalogued under the IUCN Red List® but is classified as endangered under the Indian National Threat Assessment (Kumar & Ved 2000).

Mapping and sample collection

Between 2009 and 2011, we surveyed and mapped (see Supporting information for details) all adult *D. malabaricum* trees across an area of 216 km^2 (Fig. 1) with a Garmin 60CSx handheld global positioning system (accuracy of five metres) and sampled leaf or cambium tissue from each tree for subsequent DNA extraction and genotyping. Of all trees recorded, only one was below 20 cm diameter at breast height (DBH), which had a DBH of 8.5 cm. This tree appeared in the paternity analysis twice as the most likely father. Therefore, all adult trees found were considered as reproductively mature. Of the 235 adult trees, 223 were found in 35 sacred groves, and the remaining 12 trees were found in coffee plantations. Nine of the coffee plantation trees were within 300 m of the adjacent forest fragments and

were included as part of the same 'population' of adult individuals. The remaining three trees were not assigned to any forest patch as they were isolated by more than 1 km from the nearest conspecifics in a forest patch.

To collect seedlings and saplings, we established in 2009 sixty-eight 20 × 20 m plots in 19 groves. The groves were selected to include the entire range of grove sizes, as defined by area and number of adults within each grove (ranging from one adult to 30 adults and an area of 0.63–14.39 ha). Because seedlings are predominantly found near fruiting trees, we located 23 of these plots under selected fruiting trees to assure a sufficient number of seedling samples. To account for lower densities of seedlings dispersed away from fruiting trees, the remaining 45 plots were located randomly across the 19 groves, with at least one randomly positioned plot in each sacred grove. In each plot, leaf samples from up to 20 recently germinated seedlings and from all saplings were collected if available. A total of 488 seedlings (<50 cm in height) and 119 saplings (>50 cm and <150 cm in height) were sampled from the 19 groves.

To ensure complete certainty of maternal origin of progeny, we sampled seeds directly from the canopies of 26 fruiting mother trees in 17 groves during the 2010 fruiting for paternity analysis. These trees were chosen to represent the full range of spatial isolation that occurs within our study area. On average, 22 seeds were sampled per mother tree with a maximum of 24 seeds per mother tree sampled from different positions in the canopy. In total, 566 seeds from 225 fruits were collected. The seed samples were carefully dissected to obtain pure embryo tissue (see Table S1, Supporting information for an overview of the sampling sites, the sampling plots and samples). This sampling strategy allows a detailed evaluation of pollen dispersal in a single flowering event (seed) and includes variation in the effective breeding populations over multiple reproductive events (seedlings and saplings).

Genetic analysis

All samples were dried and stored in silica gel before processing. From the lyophilized tissue, genomic DNA was extracted using a cetyltrimethylammonium-bromide extraction method (Sambrook *et al.* 1989) in the conservation genetic laboratories of the Ashoka Trust for Ecology and Environment (ATREE) and in the laboratories of the School of Ecology and Conservation at the University of Agricultural Sciences in Bangalore.

The DNA samples were genotyped at eleven nuclear microsatellite markers using the primers Dysmal 01, Dysmal 02, Dysmal 03, Dysmal 07, Dysmal 09, Dysmal 13, Dysmal 14, Dysmal 17, Dysmal 18, Dysmal 22 and Dysmal 26 (Molecular Ecology Resources Primer Development Consortium *et al.* 2010). Each polymerase chain reaction (PCR) contained 1.3 μL of DNA template, 2.06 μL 5× GoTaq Buffer (Promega), 0.62 μL $MgCl_2$ (25 mM), 2.06 μL dNTP's (2 mM each), 0.18 μL BSA (10 mg/mL), 1.03 μL of each forward and reverse primer (2 μM), Taq Polymerase (Promega) (5 U/μL) and 1.96 μL of water.

PCRs were run with two different touchdown protocols depending on the annealing temperatures of 56 and 50 °C. Both protocols start with an initial denaturation at 94 °C for 3 min followed by 14 cycles with 94 °C for 30 s, 60 or 54 °C for 45 s decreasing every cycle by 0.5 °C, respectively, 0.4 and 72 °C for 1 min. After the first 14 cycles with decreasing temperature, another 29 cycles with a stable annealing temperature of 56 or 49 °C followed, ending with a final extension of 7 min. PCRs were run on peltier thermo cyclers (Sensoquest Labcycler and BioRad Dyad). Fragment analysis was performed on an ABI3730 capillary sequencer (Applied Biosystems). Fragment sizes were scored using GeneMapper 3.5 (Applied Biosystems) relative to a LIZ 500 HD size standard.

Among the 11 microsatellite loci, we detected between 4 and 24 alleles and a total of 132 alleles in 235 adult samples. We evaluated the frequency of null alleles using two methods. Using CERVUS, only one locus Dysmal 13 showed a low frequency of null alleles (CERVUS is 0.083). Applying the software MICRO-CHECKER (Van Oosterhout *et al.* 2004) revealed a low frequency of null alleles in an additional four loci (Dysmal 02, 2%; Dysmal 09, 6%; Dysmal 18, 5% and Dysmal 26, 3%). Observed heterozygosity only significantly deviates from Hardy–Weinberg at Dysmal 13. Given the large number of alleles plus the overall low rate of parent offspring mismatches (1.5%), we consider these loci are very unlikely to introduce significant bias to the multilocus analysis. There was no evidence of significant linkage disequilibrium across loci.

Evaluating genetic diversity and genetic differentiation

We calculated for each life stage (adults, saplings, seedlings, seed) mean number of alleles (N_a) and observed and expected heterozygosity (H_o and H_e) with GenAlEx 6.41 (Peakall & Smouse 2006). Allelic richness (R_s) was estimated with FSTAT 2.9.3.2 (Goudet 1995) using 1000 permutations.

It was unknown if the adult trees in the study area are remnants of previously continuous panmictic population or historically distinct subpopulations. Therefore, we applied Bayesian cluster analysis with the admixture model provided in Structure 2.3.3 software (Pritchard *et al.* 2000); this has the advantage that it makes no prior assumption about populations. To investigate

whether differentiation has increased, we applied the same analysis to the sapling and seedling cohorts. Details of the Structure analysis are provided in Appendix S1, Supporting information.

Evaluating FSGS and historic gene dispersal

To determine the level of FSGS, we investigated spatial autocorrelation between all pairs of samples at each different distance class and the relatedness coefficient (r) and kinship coefficient (F) (Loiselle *et al.* 1995) with GenAlEx 6.4.1 (Peakall & Smouse 2006) and SPAGeDI 1.3a (Hardy & Vekemans 2002). Separate analyses were conducted for the adults and for the pooled seedling and sapling samples (hereafter called progeny samples). The seedling and sapling samples were pooled to ensure sufficient number of pairs over each distance class. The distance classes were chosen to ensure a minimum of 100 sample pairs at each distance class in each cohort. Within the first 100 m, we defined four intervals of 25 m, between 100 and 200 m two 50-m intervals, followed by a 100-m interval, and then from 300 m, we doubled the intervals from one interval to the next up to 6500 m (resulting in a total of 13 distance classes). Pairwise relatedness coefficients were computed relative to the common allele frequency of the adult and progeny samples. To compare the intensity of the FSGS between the adults and the progeny, we calculated the Sp statistics as $\mathrm{SP} = -\hat{b}_F/[1-\hat{F}(1)]$, where $-\hat{b}_F$ is the regression slope of the kinship coefficient on the natural logarithm of the distance and $\hat{F}(1)$ the mean kinship coefficient between individuals within the first distance interval (0–25 m) as described by Vekemans & Hardy (2004). To test for significant differences of FSGS across age classes and difference in the kinship among distance classes, we applied the nonparametric heterogeneity test specified in (Smouse *et al.* 2008) and carried out the analysis in the program GenAlEx 6.4 (Peakall & Smouse 2006). The heterogeneity test provides a multiclass test criterion (ω) to test the hypothesis that the entire correlogram is 'flat' against the alternative that it is not. To compare the correlograms at each distance class, we applied the single-class test statistic (t^2). The resulting P-values were adjusted with a Sequential Bonferroni correction specified in Rice (1989).

To investigate whether overall gene dispersal has changed since the adult trees established, we also estimated for the adult samples and the progeny samples the historic gene dispersal distances (σ) based on FSGS with SPAGeDI (Vekemans & Hardy 2004). Estimates of σ require information on the effective density of adult trees (D_e). Because this is unknown and estimates of σ are sensitive to the accuracy of D_e estimates, we used a range of values (D; $D/2$; $D/4$; $D/10$), where D = the estimated census density across all sacred groves (following Born *et al.* 2008).

Evaluating patterns of contemporary pollen dispersal

To investigate contemporary pollen-mediated gene flow, we applied a paternity analysis to 566 open pollinated seed collected from 26 individual mother trees (mean n = 22 per mother tree). We assigned paternity of the embryo samples using the delta maximum-likelihood method implemented in Cervus 3.0.3 (Marshall *et al.* 1998). The exclusion probabilities are for the first parent 0.996 and 0.999 for parent pairs. Simulations of paternity were run based on the multilocus genotypes of all the adult samples using the following parameters: 10 000 simulated offspring, all 235 adult trees serve as candidate fathers (allowing for selfing), the minimum number of matching loci was set at six, error rate of mistyped loci was set at 0.01% and 97% of the loci genotyped. The proportion of candidate fathers sampled was estimated to be 95%. The significance threshold for paternity assignments was based upon the 95% confidence level of the critical delta logarithm of odds score. For each assignment, the Euclidean distance between the mother tree and the most likely father was measured, representing the pollen dispersal distance.

To investigate the effect of local conspecific tree density on pollen dispersal, we grouped the mother trees based on the number of trees within 500 m. This threshold of 500 m assures for each mother tree that all conspecifics within the same grove are recognized but accounts for the rare cases for higher local densities if other conspecifics are in nearby groves. Mother trees were grouped into the following density classes: (i) six mother trees as low density with less than five conspecific trees within 500 m (average = 2.8 trees, median = 2.5 trees), (ii) 18 mother trees as high density with six or more conspecific trees within 500 m (average = 13.2 trees, Median = 10 trees) and (iii) two singleton mother trees were analysed separately.

We calculated the genetic relatedness among assigned parental pairs using the kinship coefficient (F) derived from SPAGeDI. Because the genetic relatedness of progeny can be influenced both by the kinship of parent pairs and by the number of sires in a progeny array, we also calculated the effective number of pollen donors per seed array. The number of effective pollen donors is the inverse of correlated paternity that we calculated by the fraction of full-sibs within each seed array (Ritland 1989; Hardy *et al.* 2004) obtained from the paternity analysis. The kinship of parent pairs, the frequencies of pollen dispersal distances, the number of pollen donors per seed array, the effective number of pollen donors per seed array and the corresponding

kinship of the embryo samples were compared among the density groups. To account for the nestedness of the seed samples within the mother trees, we tested for differences in mean kinship coefficients per seed array using a Wilcoxon rank sum test.

Variance in male reproductive success is important because it not only influences the patterns of pollen dispersal, but also genetic drift (Klein *et al.* 2008). To investigate the importance of varying male reproductive success in *D. malabaricum*, we estimated fecundity variance of pollen donors – the ratio of observed and effective male reproductive density (d_{obs}/d_{ep}) – using the Bayesian approach developed in Klein *et al.* (2008) with MEMM (v1.1) software (Klein *et al.* 2011). We modelled the individual fecundities using a gamma distribution including two important covariates (DBH and number of trees within 500 m). The software uses a Markov Chain randomization. We set the process starting values for the variance of (gamma-) male fecundities (σ^2), dispersal distance (δ), shape parameter (b), migration rate (m) and selfing rate (s) as (σ_0, δ_0, b_0, m_0, s_0) = (2, 100, 1, 0.5, 0.05), respectively. The simulation was run after a burn-in period of 10 000 iterations for a total of 50 000 iterations with a thinning parameter of 10 (used only for the individual fecundities).

Results

Genetic diversity and genetic differentiation in adult and juvenile life stages

To investigate the effects of habitat fragmentation on population genetic diversity, we compared diversity metrics among different life stages (adults, saplings, seedlings and seed). Table 1 provides an overview on the genetic variability across the life stages. We observed no significant differences in genetic diversity across different life stages in comparisons over all samples. The Structure analysis for the adult samples indicates the most likely clustering solution is four genetically homogenous clusters. This clustering solution shows little congruence with the geographical location of the sampling sites (Fig. S1, Supporting information), suggesting *Dysoxylum malabaricum* adults form one continuous panmictic population. The juvenile cohorts showed much clearer evidence of genetic structure (Fig. S1b,c, Supporting information). The most likely clustering solution for the sapling samples is determined to be seven. The clusters of genetically homogenous saplings corresponds in six cases to the grove of sample collection; still the majority of the samples do not cluster according to their location and therefore seem to belong to an admixture group. In the seedlings, the most likely number of clusters could not be determined unambiguously. The delta K value used to determine the most likely clustering showed one major peak at 15 clusters and additional peaks at 6 and 8 clusters. Independent of the assumed number of genetically homogenous clusters, the bar plots show an obvious clustering of the seedlings according to the different groves and around 10 sampling sites cluster as nearly pure stands.

The estimates of historic gene dispersal (σ) in adults derived from SPAGeDI (Hardy & Vekemans 2002) ranged from 171 to 804 m depending on the value of D applied. Applying an estimate of density based upon the entire study area (D') rather than based upon grove estimates (D) did not converge. Values in juveniles showed similar values with no significant difference among cohorts (based on a confidence level of 2 × the standard errors); see Table 2.

Fine-scale spatial genetic structure

Significant FSGS is observed in adult trees with kinship coefficients significantly greater than zero at all distance classes up to 900 m (Fig. 2). The intensity of FSGS based upon the Sp-statistic is 0.0107 (SE = 0.00087), with a slope (b) on the ln distance of −0.0107 (SE = 0.0009). Significant and more intense FSGS was observed in the juvenile cohorts within the first 500 m (Fig. 2), with a Sp-statistic (Sp = 0.0238, SE = 0.00146) and a slope (b) on the ln distance of −0.0206 (SE = 0.0013). The heterogeneity test confirms that FSGS is significantly different among the adults and juvenile cohort with relatedness significantly

Table 1 Genetic diversity parameters in *Dysoxylum malabaricum* across life stages

Cohorts	N	N_a	H_o	H_e	R_S
Adults	235	12.00 (±1.991)	0.628 (±0.061)	0.666 (±0.066)	10.90 (±1.72)
Saplings	119	10.09 (±1.480)	0.606 (±0.058)	0.659 (±0.071)	10.13 (±1.45)
Seedlings	488	11.36 (±1.739)	0.615 (±0.057)	0.668 (±0.065)	10.08 (±1.48)
Embryos	566	10.82 (±1.536)	0.592 (±0.059)	0.664 (±0.068)	9.69 (±1.31)

± indicates the standard error.
N, number of samples; N_a, mean number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; R_S, allelic richness based on 117 samples.

Table 2 Estimates of historic gene flow distances (σ) in *Dysoxylum malabaricum* across life stages

Density estimate	Density (adults per ha)	σ (m) Adults	σ (m) Juveniles
D	1.72	171 (±15)	202 (±29)
D/2	0.86	217 (±29)	241 (±65)
D/4	0.43	374 (±44)	389 (±131)
D/10	0.17	804 (±196)	714 (±432)
D′	0.01	4741 (±NC)	NC

± indicates the standard error
D, census density within habitat patches; *D*′, census density across the entire study area; NC, no convergence.

greater in juvenile pairs within the first six distance classes (<200 m apart) and significantly lower in the two distance classes between 300 and 900 m. The multiclass criterion (ω) shows that the overall correlograms differ significantly (ω = 73.46, P = 0.001).

Genetic connectivity of individual trees and habitat patches

The paternity analysis, used to estimate contemporary pollen flow, assigned 92% (N = 518) of the genotyped embryos with a confidence level of 95% to 108 unique pollen donors. The remaining 8% (N = 46) could not be assigned, even at the relaxed 85% confidence level. Only two selfing events were detected in all the assigned progeny (0.4%). The mean pollen dispersal distance was 1.33 km with a median of only 97 m. Seventy-eight per cent (N = 404) of the observed pollination events in progeny arrays occur within groves and do not go beyond 290 m (Fig. 3a). The pollen flow between the groves accounted for 22% (N = 114) of all pollination events. Intergrove pollination events start at 420 m and reach a maximum of 23.6 km (Fig. 3b). We detected a total of 47 (9%) pollen dispersal events beyond 5 km. Investigating the parentage of seedlings using Cervus, we could assign 66% (321) of the genotyped seedlings (n = 488) to parent pairs with a 95% confidence level. We detected seven selfed seedlings (2.2%). A total of 267 of the seedlings (83%) had both most likely parents within the same grove, demonstrating that the majority of realized pollen dispersal events were within groves. Fifty-three (17%) seedlings were the result of intergrove pollen dispersal events, and one seedling had both parents in two different groves from the one in which it was sampled. The estimates of overall male fecundity variance reveal a high mean d_{obs}/d_{ep} ratio of 12 (median, 11; 95% range, 5.3–24). Ten paternal trees are identified as contributing disproportionally to the effective pollen flow in the progeny arrays (see Fig. S3, Supporting information for details) while most trees showed a low fecundity. The 10 trees identified by MEMM as most fecund sired, according to the Cervus analysis, 200 seeds with an average pollen dispersal per tree of 100 m (median = 90 m). All ten of these trees had a DBH of more than 71 cm, and eight of them were within stands of mother trees classified with a high local density of conspecifics.

Effect of local tree density on patterns of contemporary pollen dispersal distance and relatedness of parent pairs

We compare the mean pollen dispersal, frequency of short-distance pollen dispersal and relatedness among parent pairs from the paternity analysis, classified according to their local density of conspecifics around each mother tree. These results are summarized in Table 3. The mean pollen dispersal distance of mother trees in high-density patches was twice that of mothers in low-density patches, and the proportion of mating events at short distance (<100 m) was almost double in the low-density patches compared to the high-density patches (73% vs. 46%, respectively, Table 3). Pollen dispersal was substantially higher in the two single isolated mother trees with an average pollen dispersal distance of 6.5 km (SE = 1212 m, median = 5316 m). The average kinship coefficient of the parent pairs for seed from high-density patches is low 0.061 (SE = 0.0053), and in the single mother trees, the mean kinship coefficient of the parent pairs is close to zero (0.005; SE = 0.0135). In contrast, mean kinship coefficient among parent pairs for progeny from mother

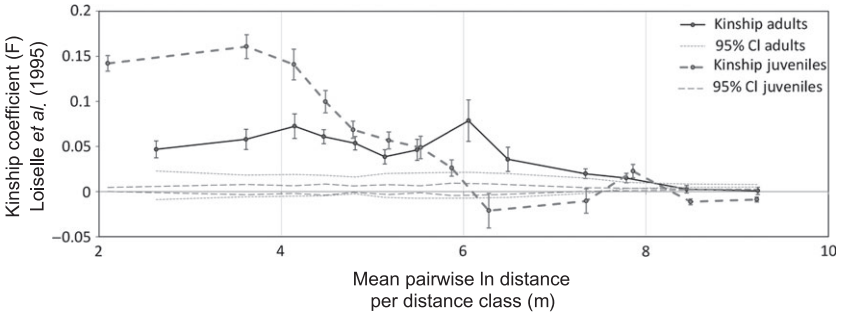

Fig. 2 Fine-scale spatial genetic structure of *Dysoxylum malabaricum* for the adult cohort (solid black line with points) and the pooled seedling and sapling cohort (dashed dark grey line with points). Error bars display standard errors based on Jackknifing over loci. The upper and lower 95% confidence levels are given for the adults (grey fine dashed line) and the juveniles (grey dashed line).

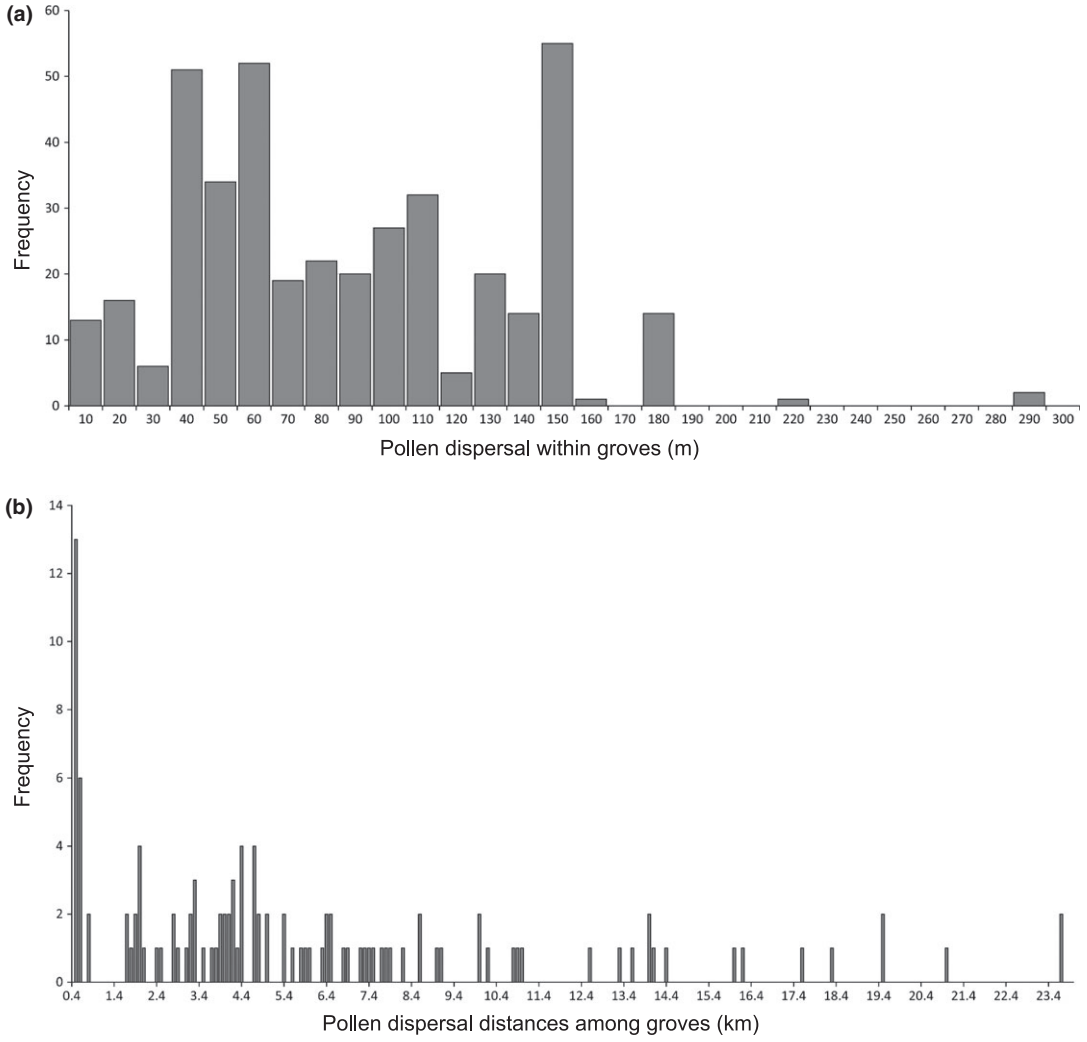

Fig. 3 Frequency distribution of pollen dispersal distances in *Dysoxylum malabaricum*. (a) Frequency distribution of within-grove pollen dispersal events in 10-metre distance classes from 0 to 300 m, $N = 404$. (b) Frequency distribution of intergrove pollen dispersal events in 200-metre distance classes from 0.4 to 24 km, $N = 114$.

Table 3 Summary table of pollen dispersal in *Dysoxylum malabaricum* and relatedness among mating individuals with mother trees with contrasting local tree density

Density class	Mother trees	Mean number of trees within 500 m (SE)	Mean pollen dispersal (median)	Proportion of mating events <100 m (*N*)	Mean relatedness of mates (SE)	Mean number of effective sires (SE)
High density	18	13.2 (1.6)	1205 m (106)	46% (163)	0.061 (0.005)	6.4 (1.4)
Low density	6	2.8 (0.4)	600 m (56)	73% (99)	0.127 (0.006)	1.6 (0.3)
Isolated trees	2	0	6525 m (5316)	0% (0)	0.005 (0.014)	54.6 (50.3)

SE, standard error.

trees in low density patches is significantly greater with mean kinship coefficients twice that of high-density patches 0.127 (SE = 0.0055), see Table 3 and Fig. 4. The number of effective pollen donors follows the same pattern: The mean number of effective sires is 6.4 (SE = 1.4) in the seed arrays from trees in high-density patches, 1.6 (SE = 0.3) sires in the seed arrays collected in low-density patches and in the two isolated trees 105.0 and 4.3 effective fathers (Table 3 and Fig. S2, Supporting information).

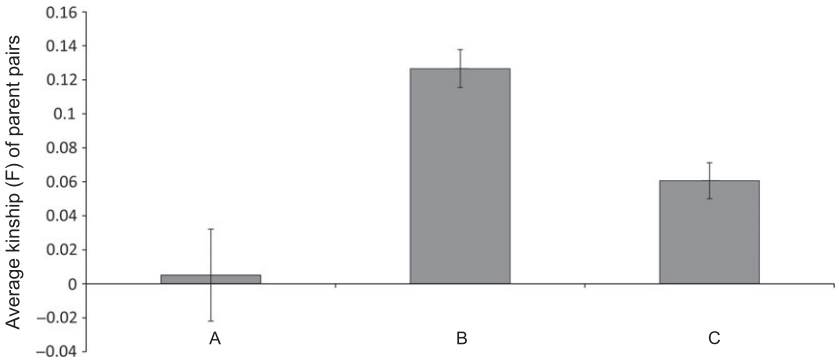

Fig. 4 Average kinship coefficients (Loiselle *et al.* 1995) in *Dysoxylum malabaricum* parent pairs of seed from mother trees with contrasting isolation. A: Seed from single isolated trees ($N = 32$). B: Seed from trees with ≤ 5 trees within 500 m ($N = 135$). C: Seed from trees with ≥ 6 trees within 500 m ($N = 349$). The error bars indicate two times the standard error.

Discussion

This study focuses on a tropical tree species confined to small (sacred grove) forest patches that are under increasing threat of exploitation and degradation. Fragmentation across the Western Ghats has been exacerbated by forests conversion to tree plantations, agriculture, and coffee and tea estates in the past 40 years (Jha *et al.* 2000; Puyravaud *et al.* 2010). Our results indicate that the surviving *D. malabaricum* trees contain substantial genetic diversity and that similar levels of diversity are present in juvenile cohorts which post-date fragmentation. Gene flow by contemporary pollen dispersal is extensive across this complex landscape mosaic, as indicated by paternity analysis and as realized pollen dispersal by parentage analysis. However, our results suggest that low local tree density coupled with significant fine-scale genetic structure can lead to elevated mating between related individuals and increased likelihood of genetic drift due to high variance in male fecundity. These processes are likely to gradually erode genetic diversity in this species. Below, we discuss the evidence to support these conclusions and the implications for the debate on forest fragmentation genetics in general.

Does fragmentation lead to genetic erosion or increased genetic differentiation?

Estimates of genetic diversity (mean number of alleles, H_e and allelic richness) over all samples do not differ significantly across the different life stages, indicating that genetic diversity is maintained within the study area. This is in contrast to many other fragmented woody plant species which show reduced genetic diversity in juvenile cohorts (Vranckx *et al.* 2012). A likely explanation is the extensive pollen dispersal which enhances the effective population size in remnant patches and results in the maintenance of the overall gene flow distance as shown by our estimates of historic gene dispersal. Even though most pollen is transported within forest patches, the paternity analysis reveal that about 22% of pollen transport occurs between groves. This is consistent with other empirical studies of pollen flow in tree species in fragmented landscapes, which demonstrate robust pollen dispersal (e.g. White *et al.* 2002; Dick *et al.* 2003; Jha & Dick 2010; Lander *et al.* 2010).

The increase in genetic differentiation between cohorts as indicated by the Bayesian clustering suggests that fragmentation could be leading to an increase in genetic differentiation in more recently established cohorts. This increase in genetic differentiation suggests that realized gene flow may be insufficient to counteract effects of drift especially when local densities are decreasing. Similar patterns have been observed in other tropical tree species where pollen is dispersed over relatively long distances and genetic differentiation increases among cohorts (Kettle *et al.* 2007; Rosas *et al.* 2011). In this context, fecundity variance (Klein *et al.* 2011; Moran & Clark 2011) and temporal isolation due to asynchronous flowering (Fuchs *et al.* 2003) may play an important role as both effects amplify genetic drift. Although we cannot determine the effects of asynchronous flowering, our estimates of fecundity variance demonstrate a disproportional contribution of some paternal trees in our study system, suggesting that genetic drift is also an important factor in increasing genetic differentiation (Klein *et al.* 2008). Indeed, in the 10 most fecund males (identified with MEMM v1.1) 97% of mating events are within grove (data not shown).

Does local density influence patterns of contemporary pollen dispersal?

When trees show a clumped distribution, pollen movement is often dominated by the closest conspecifics (Garcia *et al.* 2005; Pluess *et al.* 2009). The importance of spatial distribution of adult trees for pollen flow and nearest neighbour mating has already been well established (Stacy *et al.* 1996; White *et al.* 2002; Dick *et al.* 2003; Garcia *et al.* 2005). However, these other studies have not investigated the implications of these mating patterns in terms of elevated mating between related

individuals or inbreeding. In our study, the proportion of short-distance mating events in *D. malabaricum* is substantially greater when few conspecifics are present at the local scale; this leads to an increase in the relatedness among mating pairs and a reduction in the number of effective pollen donors. Pollinator foraging seems to favour mating among trees within the same forest patch, which in larger stands includes more effective pollen donors, less related trees and longer distances within the fragment. In contrast, isolated mother trees show a very different pattern in their mating events. The two isolated mother trees in our study receive pollen from across our research area with average distances of 4.5 km (N = 17) and 8.8 km (N = 15) and from multiple fathers (7 and 14, respectively). This demonstrates that single trees have the potential for effective mating over long distances and sample a heterogeneous pollen pool.

Implications of FSGS for inbreeding in highly fragmented landscapes

We observed significant FSGS in adult populations of *D. malabaricum*. This aggregation of more related individuals at short distances may be driven by several factors including limited pollen and seed dispersal, population density, mating system (Vekemans & Hardy 2004), regeneration dynamics after small- and large-scale disturbance (Premoli & Kitzberger 2005; Kettle *et al.* 2011), maternal correlated seed clumps caused by the foraging pattern of frugivores (Torimaru *et al.* 2007; Sezen *et al.* 2009) and colonization history (Jones *et al.* 2006). The specific drivers of FSGS in *D. malabaricum* are not the focus of this study, but it is clear that habitat fragmentation is predicted to exacerbate many of the drivers of FSGS, such as seed dispersal (Cramer *et al.* 2007; McConkey *et al.* 2012). This implies that FSGS may become more intense in tropical tree species surviving in highly fragmented agro-forest landscapes. Similar processes have been observed in chronically fragmented temperate tree species despite effective gene flow (Dubreuil *et al.* 2010). If pollen-mediated gene flow is dominated by near neighbours in concert with intense FSGS, this will lead to elevated inbreeding over time (Hirao 2010; Chybicki *et al.* 2011) as shown in the present study with the increased relatedness of parent pairs and the reduced number of effective pollen donors in low-density stands.

Furthermore, we observe a greater intensity of FSGS in juveniles compared to adults (as indicated by the Sp-statistic, the slope estimates and the Heterogeneity test). An increase in mortality over time through self-thinning of more related individuals could contribute to this pattern. Evidence from the tropical tree *Caryocar brasiliense* suggests that competition is greater among more related individuals (Collevatti & Hay 2011). Interestingly, we observe significantly lower relatedness among juvenile pairs within the 300- to 900-metre distance class than in the adult pairs at the same distance. This pattern corresponds to the spatial scales over which gene dispersal (by seed and pollen) appears to have been reduced by fragmentation in *D. malabaricum* over recent decades, as indicated by the increased genetic differentiation between fragments in juvenile cohorts. If the observed patterns of FSGS in the current juvenile cohort of *D. malabaricum* persist over time, or the density of adult trees is further reduced by illegal logging and habitat degradation, then our results suggest that mating between related individuals will increase. The combination of more short-distance mating events and high FSGS suggests that tropical tree species with the capacity for long-distance pollen dispersal may still be vulnerable to forest fragmentation genetics, such as elevated inbreeding.

Management implications

Our results provide solid evidence that pollen dispersal can and does occur over large distances in highly fragmented agro-forest landscapes. However, as forests become more fragmented, inbreeding due to increased short-distance mating events and genetic drift are likely to be exacerbated. Whether the increased frequency of mating between related individuals will result in deleterious consequences for seedling growth or survival in *D. malabaricum* remains to be determined. Considerable evidence already exists to support the idea that increased inbreeding leads to reduced fitness across a wide range of plant species (Reed & Frankham 2003; Leimu *et al.* 2006).

Adopting a precautionary principle for the management of *D. malabaricum*, it would be prudent to maintain grove sizes >6 trees and ensure that distance between groves does not increase. It would be beneficial to assist recolonization of sacred groves by enrichment planting of *D. malabaricum* and other native tree species with similar reproductive ecology. Our study certainly indicates that *D. malabaricum* would benefit from a reduction in the distances between groves to below 1 km. This would enhance pollen dispersal at intermediate distances (0.5–1 km), which would further reduce inbreeding. Although over our entire study area, genetic diversity remains high, private alleles are present in only 15 of the surveyed 35 forest patches (of which five occur in patches with less than five trees). This diversity is therefore vulnerable to ongoing habitat encroachment and illegal logging. Artificial seed exchange or transplanting of nursery-raised seedlings

among groves would help to counteract the observed genetic differentiation at the landscape level.

The paternity analysis demonstrates that isolated trees have the potential to attract pollinators over long distances, a phenomenon observed in several other fragmented tropical trees (e.g. White *et al.* 2002; Dick *et al.* 2003; Jha & Dick 2010; Lander *et al.* 2010). The maintenance of native trees in coffee plantations should therefore be promoted. This not only enhances genetic connectivity across the landscape, as the progeny from single trees are sired by unrelated trees, but presents a currently unexploited genetic resource. Establishment of native tree seedlings in coffee plantations is hindered by regular weeding. These single adult trees offer an important (unexploited) source of genetically diverse and outbreed seeds which could be used for forest restoration (Ottewell *et al.* 2010).

Fragmentation is accelerating across the tropics with an abundance of tropical tree species threatened with extinction (Newton & Oldfield 2008). Many of these tropical tree species are likely to exhibit limited seed dispersal (McConkey *et al.* 2012; Kettle 2012) and significant FSGS. Ensuring local tree densities remain above a critical threshold and that interfragment distances remain below a few kilometres are likely to reduce the negative genetic consequences of habitat fragmentation even in species with the potential for long-distance pollen-mediated gene flow.

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Data accessibility

Sample locations and microsatellite data: DRYAD entry doi: 10.5061/dryad.3ck30.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Sampling sites and sample collection of *Dysoxylum malabaricum*.

Table S2 Overview on *Dysoxylum malabaricum* individuals defined as mother tree showing: Adult trees within grove, seed array assignments, pollen donors, effective pollen donors, pollen immigration, pollen dispersal distances, kinship coefficient of parent pairs and degrees of isolation (sorted by classification of isolation degree).

Fig. S1 Fraction of ancestry based on Bayesian clustering of the *Dysoxylum malabaricum* age cohorts with Structure software for (a) the 235 adult samples for four assumed clusters, (b) the 119 sapling samples for seven assumed clusters and (c) the 488 seedling samples for 8 assumed clusters. The numbers below the bar plots indicate the sampling sites.

Fig. S2 Boxplot of the number of effective pollen donors (Nep) per mother tree classified according to the local density of conspecific trees within 500 m.

Fig. S3 Individual fecundity of all adult trees, showing that most trees contribute little pollen and few trees disproportionally more pollen.

Appendix S1 Survey methods for the inventory of *Dysoxylum malabaricum* within the study area.

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