Doctoral Thesis

Fragmentation Genetics of Tropical Tree Species in an Agro-Forest Landscape

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Publication Date:
2013

Permanent Link:
https://doi.org/10.3929/ethz-a-009937218

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FRAGMENTATION GENETICS OF TROPICAL TREE SPECIES
IN AN AGRO-FOREST LANDSCAPE

A dissertation submitted to
ETH ZURICH
for the degree of
Doctor of Sciences

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2013
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SUMMARY

Trees and forest patches are key elements of tropical agro-forest landscapes, providing a substantial contribution to global biodiversity conservation. The global trend of agricultural intensification threatens biodiversity as landscapes get simplified and tree communities become gradually impoverished. In addition to clearing and selective logging, remaining trees and populations in agricultural landscapes get spatially disrupted which can affect their reproduction via genetic and ecological processes. The response of individual tree species to fragmentation depends largely on the ability to maintain genetic connectivity which ensures mating and counteracts genetic drift and inbreeding. The genetic consequences of habitat fragmentation threaten species viability by reducing reproductive output and eroding their evolutionary potential to adapt to changing environments.

In this thesis the effects of population fragmentation on gene flow and on reproduction of two tree species were investigated in the agro-forest landscape of Kodagu District, South India. These investigations focused on *Dysoxylum malabaricum* (Meliaceae) and *Vateria indica* (Dipterocarpaceae), two rare endemic tree species of the Western Ghats biodiversity hotspot. *Dysoxylum malabaricum* is dispersed by the Malabar Grey hornbill (*Ocyceros griseus*) and generally occurs within the study area as small groups of trees confined to widely separate small forest patches. In contrast the heavy single seed of *V. indica* is expected to be dispersed by gravity only. Within the study area *V. indica* is largely confined to river banks were it can occur abundantly and in exceptional cases as dense stands within the agricultural matrix.

The ultimate aim of this thesis is to investigate the genetic consequences of habitat fragmentation in both species. These consequences are shaped by pollen and seed dispersal which is investigated with a combination of indirect estimates and direct estimates based on paternity and parentage analysis. Whether inbreeding due to fragmentation leads to reduced growth performance was investigated using common garden nursery trials. In addition to genetic consequences of spatial isolation, reproduction of tree species may be additionally affected by reduced habitat patch quality. Therefore indicators of habitat patch degradation were related to seedling densities of *D. malabaricum*.

The results demonstrate that *D. malabaricum* has the capacity for maintaining genetic connectivity by long distance pollen dispersal exceeding distances greater than 5 km. Isolated trees receive heterogeneous pollen from distant trees, whereas trees occurring in clumps receive pollen predominantly from conspecifics at short distances which increases inbreeding. This
effect becomes more pronounced as the local density of trees declines. The parentage analysis of *D. malabaricum* seedlings demonstrate, that seed migration events between forest patches in this landscape are extremely rare and that most of the seeds are dispersed close to the maternal tree. This is contrary to the expected dispersal potential of a large bird species.

Considering habitat degradation together with genetic effects of fragmentation in *D. malabaricum* demonstrates that both processes affect reproduction. Fragmentation causes reduced seedling vigour through inbreeding and habitat degradation reduces seedling densities. The co-occurrence of both effects makes interactions between genetic effects and adverse local conditions likely.

Parentage analysis in *V. indica* revealed much lower rates of selfing in the seedlings sampled in the wild compared to the seed and nursery raised seedlings, suggesting an effective and early acting selection under natural conditions. The nursery experiment confirms the finding that selfed seedlings show reduced vigour. The results suggest that such selection coupled with rare realized long distance pollen flow in *V. indica* may help maintain genetic diversity and reduce inbreeding depression in an isolated population.

Although the vital role of trees and forest structures for supporting biodiversity in human dominated landscapes is now widely recognized, the value of such landscapes to maintain the genetic diversity receives much less attention. Over all the findings of this thesis highlight the potential of complex agro-forest landscapes to harbour genetic diversity of trees. The investigations of pollen and seed dispersal demonstrate the importance of understanding these processes to implement adequate management that ensures persistence of trees in such landscapes. This knowledge is essential to maintain tree species as well as their genetic diversity in human dominated landscapes which is of fundamental importance to sustain both biodiversity and ecosystem services they provide.
ZUSAMMENFASSUNG

heterogenen Pollen von weit entfernten Bäumen, wohingegen geklumpt auftretende Individuen Pollen vorwiegend von den nächsten Bäumen erhalten, was die Inzucht erhöht. Dieser Effekt wird ausgeprägter, wenn die lokale Dichte der Bäume abnimmt.


Die Elternanalyse in *V. indica* zeigt viel geringere Selbstbestäubungsraten in Sämlingen, welche in der Natur gesammelt wurden, als in Samen und Sämlingen, welche in der Baumschule aufgezogen wurden. Das lässt auf eine wirksame und frühe Selektion unter natürlichen Bedingungen schliessen. Das Experiment in der Baumschule bestätigt das reduzierte Wachstum der selbstbestäubten Sämlinge. Die Resultate weisen darauf hin, dass in einer isolierten *V. indica* population die Selektion zusammen mit seltener aber realisierter Pollenausbreitung über lange Distanzen dazu beiträgt, die genetische Vielfalt zu erhalten und die Inzuchtdepression zu reduzieren.

Auch wenn weitgehend anerkannt ist, dass Bäume und Waldstrukturen in anthropogenen Landschaften für die Bewahrung der Biodiversität eine entscheidende Rolle spielen, wird dem Wert solcher Landschaften für die Erhaltung der genetischen Vielfalt wenig Beachtung geschenkt. Über die ganze Arbeit gesehen betonen die Resultate das Potential solcher komplexen Agroforstlandschaften die genetische Vielfalt von Bäumen zu erhalten. Die Prozesse der Pollen- und Samenausbreitung zu verstehen ist wichtig, um adäquate Massnahmen zum Erhalt von Baumarten umzusetzen. Dieses Wissen trägt entscheidend dazu bei, dass Baumarten und deren genetische Vielfalt in vom Mensch dominierten Landschaften fortbestehen und die Grundlage für die Biodiversität und die Ökosystemleistungen solcher Landschaften bilden.
CHAPTER 1

General introduction

| The vital role of trees in the global biodiversity crisis

Tropical forests represent the most species rich terrestrial habitats harboring more than 60% of the known species (Dirzo and Raven 2003). Therefore conversion of tropical forest to agricultural systems is recognized as one of the major causes of current global biodiversity loss (Bradshaw et al. 2009; Pereira et al. 2010). Current rates of species extinction are thought to exceed the extinction rates of the “big five” historical mass extinction events where 75% of the species were lost (Barnosky et al. 2011). Current extinction rates are estimated to be 100 to 1000 times faster than could be considered natural reaching levels expected to cause major impacts on global ecological processes with negative consequences for human well-being (Rockström et al. 2009). While such global predictions have inherently large uncertainties there is little quantitative evidence about how badly the current loss of biodiversity affects ecosystem functioning and provision of ecosystem goods and services (Purvis and Hector 2000). Additionally, the degradation of many ecosystem services, at least in the short term, can be accompanied by increased human well-being. We do not understand well the complexity and trade-offs associated with these underlying processes (Raudsepp-Hearne et al. 2010). Considering the uncertain but potentially far-reaching consequences of the current biodiversity crisis a precautionary principle demands that conservation of biodiversity remains an important societal responsibility. It has to be kept in mind that this is an anthropocentric view because the earth’s history has proven that biodiversity will recover through speciation - but recovery of biodiversity from mass extinction events is on timescales of millions of years (Sahney and Benton 2008) which is of no direct relevance for human well-being.

Contrary to the mainstream view that global biodiversity is seriously imperiled it has been projected that “Current human demographic trends - slowing population growth and intense urbanization - give reason to hope that deforestation will slow, forest regeneration through secondary succession will accelerate, and a mass extinction of tropical forest species can be avoided.“ (Wright and Muller-Landau 2006). This view has fueled a scientific debate on the future
of tropical biodiversity which was reviewed by Laurance (2007) who concluded that the tropical extinction crisis was unlikely to be significantly slowed down by these demographic trends suggested by Wright and Muller-Landau (2006). To date it seems widely accepted that loss of tropical biodiversity is of global concern (Bradshaw et al. 2009) and that for effective biodiversity conservation there is no substitute for primary tropical forests (Gibson et al. 2011). Around 11.3% of the tropical forests are explicitly designated for biodiversity protection (Schmitt et al. 2009) which on its own are not sufficient to stop global biodiversity loss (Brooks et al. 2004).

For effective biodiversity conservation it is now widely accepted that the matrix as well as the people around protected areas need to be incorporated (Harvey et al. 2008; Chazdon et al. 2009; Gardner et al. 2009). Diverse tropical landscape mosaics outside of protected areas can harbor high levels of biodiversity with trees and forest fragments recognized as key elements (Peh et al. 2006; Bhagwat et al. 2008; Perfecto and Vandermeer 2008; Anand et al. 2010; Mulwa et al. 2012). Indeed, trees in agricultural landscapes were shown to have a disproportionate positive effect on biodiversity relative to their abundance and the area they occupy (Tews et al. 2004; Manning et al. 2006; Fischer et al. 2010). Due to insufficient recruitment and agricultural intensification mature trees in such landscapes are globally declining (Gibbons et al. 2008). In forest fragments mature old growth trees are also declining because they experience elevated mortality due to uprooting, infestation by lianas and desiccation near forest edges (Laurance et al. 2000). This global trend of declining mature trees might undermine the potential for human dominated landscape mosaics to ameliorate loss of biodiversity. If complex agro-forest landscapes are to sustain both biodiversity and their ecosystem services in the future, it is imperative to manage these to ensure persistence of large old growth tree species.

**The ecological and genetic responses of trees to land use change**

Trees and forest fragments in tropical agricultural landscapes are in most situations likely to be remains of formerly continuous forests. Therefore the surrounding habitat and species composition has changed dramatically since the mature trees established. If we want to ensure the persistence of tree species we need to understand their response to changes they face in such landscapes. Generally land use change causes loss of continuous natural habitats which spatially disrupts tree populations and isolates individual trees from conspecifics within the landscape.

Species viability in remnant habitat patches has been related to habitat patch size and the degree of isolation in a number of theoretical and experimental studies (e.g. Saunders et al. 1991; Turner 1996; Debinski and Holt 2000; Thornton et al. 2011) and is of central interest
in population ecology, genetics and in conservation studies (Fahrig 2003). More specifically, fragmentation affects the reproductive output of many plant species by reducing the number of reproductive individuals and by disrupting pollination and seed dispersal processes (Henle et al. 2004; Ghazoul 2005). Individual plants in small isolated patches receive fewer pollinator and seed disperser visits which directly reduces seed production and the likelihood of seed establishment (Bond 1994, Cunningham 2000, Rodriguez-Cabal et al. 2007). Susceptibility of tree species reproductive ecology is very likely to be a function of several factors including size of the floral display, the breeding system, the pollination system, the seed dispersal mechanism, the distribution of trees through the landscape, the nature of the intervening habitat matrix (Ghazoul 2005) as well as the response to abiotic factors like drier conditions caused by edge effects (Benitez-Malvido 1998; Bruna 1999; Uriarte et al. 2010). Therefore effects on reproductive ecology and recruitment are not uniform across tree species. More severe consequences are expected for shade tolerant (Benitez-Malvido and Martinez-Ramos 2003) and animal dispersed plants (Cordeiro and Howe 2001; Montoya et al. 2008; Moran et al. 2009). These species specific responses to forest fragmentation can cause dramatic shifts in species compositions where old growth trees are replaced by lianas and early successional species (Laurance et al. 2001; Laurance et al. 2006).

The expected genetic consequences of habitat fragmentation are reduced genetic variability and increased inter-population genetic divergence due to increased random genetic drift, inbreeding and reductions of gene flow (Young et al. 1996; Lowe et al. 2005; Aguilar et al. 2008). The response of an individual tree species depends largely on the ability to maintain genetic connectivity between individuals and subpopulations which counteracts genetic drift and inbreeding (Couvet 2002; Trakhtenbrot et al. 2005). Many studies have applied paternity analysis to measure pollen flow, revealing that pollen dispersal can occur over long distance, especially in tree species occurring at naturally low densities (Petit and Hampe 2006; Dick et al. 2008). 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). Concluding from such findings a general robustness of tree species to habitat fragmentation (e.g. Kramer et al. 2008) seems inadequate because it can be expected that almost every dispersal vector has a spatial limit and therefore in every species populations can be separated by distances which cannot be bridged by dispersal events.

Indeed, there is a growing number of studies on threatened woody species which show that habitat destruction and consequently the disruption of continuous populations causes ele-
vated inbreeding (Fuchs et al. 2003; Kettle et al. 2007; Dick et al. 2008; Lobo et al. 2012), reduced reproductive output (Ghazoul and McLeish 2001; Rocha and Aguilar 2001; Cascante et al. 2002; Finger et al. 2011) and reduced fitness of the progeny (Cascante et al. 2002; Breed et al. 2012a).

Spatial restrictions for gene dispersal patterns not only determine the migration rates but also lead to local aggregation of related individuals (Vekemans and Hardy 2004). Therefore plants frequently exhibit a decrease of pairwise kinship with increasing pairwise distances, so called fine-scale spatial genetic structure (FSGS). In plant species this FSGS is driven by limited seed dispersal, pollen dispersal, population density, life-form, mating system (Vekemans and Hardy 2004), but also by gap dynamics and regeneration mode (Premoli and Kitzberger 2005) and colonization stage (Chung 2008). When gene dispersal is reduced by fragmentation the intensity of the FSGS can be expected to increase. Due to the longevity of trees such changes should become apparent when comparing FSGS of individuals which established prior to fragmentation and individuals which established after fragmentation. Tree species which have naturally restricted seed dispersal and therefore intense FSGS are likely to be more sensitive to fragmentation because it might promote genetic isolation of related individuals and consequently consanguineous matings. Indeed, there are examples of tree species where fragmentation caused increased inbreeding due to more frequent short distance mating among spatially clumped and related individuals (Stacy et al. 1996; Garcia et al. 2005; Breed et al. 2012b).

Because loss of genetic diversity and inbreeding depression are recognized as relevant forces reducing fitness and pushing species towards extinction (Keller and Waller 2002; Spielman et al. 2004; Frankham 2005; Angeloni et al. 2011) it becomes apparent that understanding gene flow of trees in human dominated landscapes is an important component to successfully manage their short and long term persistence.

| Kodagu, a diverse agro-forest landscape within the Western Ghats |

The hill chain of the Western Ghats runs along 1600 km of the west coast of India from 21°N to 6° N latitude. The Western Ghats are classified as one of 25 global biodiversity hotspots due to their extraordinary endemism and their degree of threat (Myers et al. 2000). This biodiversity hotspot is by far the most densely populated (Cincotta et al. 2000) with a long history of agricultural land uses. Agro-pastoral systems were already established as early as 3000 years B.P. (Chandran 1997). Commercial timber extraction and spice production started around 400 years B.P. with an increase in timber harvesting beginning around 200 years ago. As a result the forests of the Western Ghats have experienced extensive forest loss and fragmentation
(Menon and Bawa 1997) with remnant forest likely to be exposed to increasing anthropogenic degradation. India has already lost 80% of its native forest cover (Laurance 2007) and faces an ongoing decline of native forest of 1.5% - 2.7% per annum (Puyravaud et al. 2010). The remaining forests and forest patches are under massive pressure with an estimated 275 million people relying on forest resources, including fuel wood and fodder, for subsistence or cash livelihoods (World Bank 2006). Despite the seemingly daunting preconditions for biodiversity the Western Ghats still harbor high levels of biodiversity in very diverse landscape mosaics containing a wide variety of natural, semi-natural and agro-ecosystems (Anand et al. 2010).

Within the Western Ghats we focus on Kodagu district (also known as Coorg) which belongs to the state of Karnataka. Kodagu lies at the eastern slopes of the central Western Ghats and provides an excellent example of such a diverse agro-forest landscape which harbors great biodiversity (Bhagwat et al. 2005b; Page et al. 2010) and provides high incomes. Kodagu is with the third highest incomes one of the wealthiest districts within Karnataka (Government of Karnataka 2006). The region is still dominated by forest with an estimated forest cover of 46% (Garcia et al. 2010). Central Kodagu is under intensive land use dominated by coffee production and paddy. Commercial coffee growing in the region started in the 1850s (Bidie 1868). The expansions of coffee plantations made the district one of the major coffee growing regions of India, contributing to one third of the Indian coffee production (Coffee Board of India 2008 in Garcia et al. 2010). Between 1977 and 2007 there was more than 30% loss in forest cover while coffee production has more than doubled (Garcia et al. 2010). Nowadays one third of the land is under coffee plantations, many of which still use native trees as shade cover. The dense native shading contributes substantially to the maintenance of the high biodiversity in the region (Bhagwat et al. 2005a). Along with the expansion of the coffee plantations went an intensification of coffee production and a shift to irrigated coffee. This development has facilitated the replacement of native shade trees with fast growing exotic species (e.g. *Grevillea robusta*) that provide little shade and support minimal native biodiversity (Garcia et al. 2010).

Embedded within this agricultural matrix is a large amount of small forest fragments. The large majority of these fragments are so called ‘sacred groves’. Sacred groves are small remnants of forest conserved by the local community for worshipping (Chandrakanth et al. 2004). These forests are recognised as primary example of community based natural resource management (Bhagwat and Rutte 2006; Ormsby and Bhagwat 2010). Kodagu district harbours an exceptionally high density of one ‘sacred grove’ forest in each 300 ha (Kushalappa and Bhagwat 2001). Although 80% of the sacred groves are smaller than two hectares (Kushalappa et al. 2001) and contribute only to about 2% of the forest cover in the region (Bhagwat et
al. 2005a), they are recognised as important repositories of native biodiversity with distinct species compositions to the nearby reserve forests (Bhagwat et al. 2005b; Page et al. 2010). Sacred groves are especially important for medicinal plants which might have been selectively favoured by local communities. The density of medicinal plant species is almost twice that of ‘reserve’ forests, with nearly 40% of these medicinal plant species apparently unique to sacred grove forests (Boraiah et al. 2003). Further these sacred groves contain large trees providing vital nesting sites for the giant honey bee (Apis dorsata) which supply substantial pollinator services to the surrounding coffee plantations (Krishnan et al. 2012). Within our study area, the sacred groves were offered some legal protections by the colonial administration already in 1873, when the boundaries were delimited and coffee cultivation within their limits was banned (Pouchepadass 1990). However, this legal protection was not very effective: In the past hundred years the area of the sacred grove forests in Kodagu has declined by over 50% while their number increased from 873 to 1214 (Kalam and Thanuja 2000), most likely due to the splitting up of the groves. The loss of cultural affinity with the sacred grove forests has also led to less effective management, resulting in further degradation of the groves (Ormsby and Bhagwat 2010). Such degradation takes the form of illegal timber and wood extraction and the encroachment of the sacred groves by coffee plantations (Chandrakanth et al. 2004) but also by intensive extraction of fuel wood and non-timber forest products by the local community (Garcia C.A. and Pascal J.P. 2006). For these reasons the sacred grove forests of the region provide an ideal model system to investigate effects of forest fragmentation, where an increase of habitat patches went along with reduction of total habitat area.

**Overall aims of thesis**

Within central Kodagu the landscape development from long term moderate fragmentation to more recent large scale habitat loss and severe fragmentation allows to investigate in detail gene flow and genetic consequences of these changes for tree species. By investigating two species with contrasting seed dispersal mechanisms and distribution differing vulnerability to spatial isolation are expected. For that reason I have chosen Dysoxylum malabaricum (Meliaceae) and Vateria indica (Dipterocarpaceae), two rare endemic tree species of the Western Ghats.

Within the study area *D. malabaricum* is sparsely distributed and almost exclusively confined to the sacred grove forests where it occurs as isolated individuals up to 30 individuals. The seed of *D. malabaricum* is dispersed by the Malabar Grey hornbill (*Ocyceros griseus*). Contrastingly the heavy single seeded fruit of *V. indica* is expected to be dropped near the mother tree as there is no dispersal agent known. This species is extremely rare within the agricultural
matrix and is largely confined to river beds were it can occur abundantly. In exceptional cases *V. indica* may be found in the agricultural matrix as dense stands in sacred groves far from any river. I compare one such isolated population consisting of 85 *V. indica* trees with a continuous population along a river.

In both species I investigate how genetic diversity and spatial genetic structure might have changed over time by comparing adult life stages with juvenile life stages. Differences of genetic diversity and spatial genetic structure across life stages can shed light on the effects of spatial isolation for genetic structure of plant populations. Because gene flow is the underlying process that shapes the genetic structure we investigate in detail contemporary pollen and seed dispersal as well as historic levels of gene dispersal. In particular we compare in both species gene flow at different levels of isolation and across different life stages to detect the effect of spatial isolation. Gene flow among subpopulations of a species is an important factor to ameliorate expected loss of genetic diversity and elevated inbreeding due to fragmentation (Young *et al.* 1996). To investigate the potential fitness consequences of fragmentation induced inbreeding, we additionally recorded growth performance of seedlings grown under controlled nursery conditions.

Because habitat fragmentation is generally accompanied by additional habitat patch degradation I further investigate in *D. malabaricum* how spatial isolation and habitat patch quality affect reproductive success. Because such multiple threats commonly co-occur interactions among multiple threats are expected be major factors in pushing species towards extinction (Myers 1987; Laurance and Cochrane 2001; Brook *et al.* 2008). The overall expectation here is that habitat fragmentation, population isolation and habitat patch degradation affect in concert reproductive success via genetic and ecological processes in *D. malabaricum*.

This thesis aims to contribute to the understanding of how tree species can be managed in human dominated landscapes. Based on our findings we provide management recommendations to ensure long and short term persistence of trees in such fragmented landscapes. This is not only important for maintaining the ecological integrity of such landscapes but also to conserve valuable genetic diversity represented in remnant trees. Such trees in agricultural landscapes or degraded forests can provide valuable genetic resources which can be most important for collecting genetically diverse and locally adapted seed for effective restoration of degraded forests.
REFERENCES


References


CHAPTER 2

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

*with J. Ghazoul | G. Ravikanth | R. Uma Shaanker | C.G. Kushalappa | C.J. Kettle |

*Published in Molecular Ecology (2012), 21, 5484-5968*

**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 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 of differentiation and fine-scale spatial genetic structure (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 five kilometres. 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.
CHAPTER 2

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 and 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 and Jump 2011). Many studies have applied paternity analysis to measure pollen flow, revealing that pollen dispersal can occur over long distances, especially in tree species occurring at naturally low densities (Petit and 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 the long and overlapping generations of tree species 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. 2011) and reduced reproductive fitness (Rocha and 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. 2101; 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 bi-parental 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).

In order 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; Finger et al. 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 fine-scale spatial genetic structure in the tropical timber tree *Dysoxylum malabaricum* (Meliaceae). We intensively surveyed an area of 216 km² in Kodagu district, part of the Western Ghats biodiversity hotspot in South India (Figure 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 paper 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: (1) Fragmentation leads to genetic erosion and increased genetic differentiation. (2) Local density influences patterns of contemporary pollen dispersal (3) Short distance pollen dispersal coupled with FSGS lead to elevated inbreeding in highly fragmented landscapes. *D. 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 paper 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 and Jump 2011), however many species show increased bi-parental inbreeding in fragmented habitats (Vranckx et al. 2012). Elucidating this paradox of forest fragmentation genetics is relevant directly to 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 hot spot. In the past hundred 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 and 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 un-sampled pollen donors in continuous forest.

Dysoxylum malabaricum (Meliaceae) is a monoecious 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 one 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 (Ismail pers obs 2009). The fruit, contains 4 carpels with 2 ovules each, of which normally only one ovule develops. When mature the fruit splits along the septa into 4 sections, displaying the seed (approx. 30 mm x 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 fruit drop to the ground with their seed coat still attached, they rot quickly on the forest floor suggesting obligate zoochory (Ismail pers obs 2010).
*D. malabaricum* is a large (reaching heights in excess of 45 metres) 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 (Ismail *pers obs* 2007). The species is not catalogued under the IUCN Red List® but is classified as endangered under the Indian national threat assessment (Kumar and 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 km² (Figure 1) with a Garmin 60CSx handheld GPS (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 where considered as reproduc-

To collect seedlings and saplings we established in 2009 sixty-eight 20 x 20 metre plots in 19 groves. The groves where 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 to 14.39 hectares). 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 where 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).

Figure 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 km²). 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.

| Genetic analysis |
All samples were dried and stored in silica gel before processing. From the lyophilized tissue genomic DNA was extracted using a CTAB 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 5x GoTaq Buffer (Promega), 0.62 µl MgCl₂ (25mM), 2.06 µl dNTP’s (2mM each), 0.18 µl BSA (10mg/mL), 1.03 µl of each forward and reverse primer (2 µM), Taq Polymerase (Promega) (5U/µl) and 1.96 µl of water.

PCRs were run with two different touchdown protocols depending on the annealing temperatures of 56°C and 50°C. Both protocols start with an initial denaturation at 94°C for 3 minutes followed by 14 cycles with 94°C for 30 seconds, 60°C or 54°C for 45 seconds decreasing every cycle by 0.5°C respectively 0.4°C and 72°C for 1 minute. After the first 14 cycles with decreasing temperature another 29 cycles with a stable annealing temperature of 56°C or 49°C followed, ending with a final extension of 7 minutes. PCRs were run on peltier thermo cyclers (Sensquest 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 (Dysmal02 2%; Dysmal09 6%; Dysmal18 5% and Dysmal26 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 multi-locus analysis. There was no evidence of significant linkage disequilibrium across loci.

<table>
<thead>
<tr>
<th>Evaluating genetic diversity and genetic differentiation</th>
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</table>
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 and 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 sub populations. 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 if differentiation has increased we applied the same analysis to the sapling and seedling cohort. Details of the STRUCTURE analysis are provided in the supplementary information.

| Evaluating fine-scale spatial genetic structure and historic gene dispersal |

To determine the level of fine-scale spatial genetic structure (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 and Smouse 2006) and SPAGEDI 1.3a (Hardy and 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 metres we defined four intervals of 25 metres, between 100 and 200 metres two 50 metre intervals, followed by a 100 metre interval and then from 300 metres we doubled the intervals from one interval to the next up to 6500 metres (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 $Sp = -\hat{b}_r/(1-\hat{f}(1))$, where $-\hat{b}_r$ 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 metres) as described by Vekemans and 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 programme GenAlEx 6.4 (Peakall and Smouse 2006). The heterogeneity test provides a multiclass test criterion ($\omega$) 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 if 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 ($\sigma$) based on FSGS with SPAGEDI (Vekemans and Hardy 2004). Estimates of $\sigma$ require information on the effective density of adult trees ($D_e$). Because this is unknown and estimates of $\sigma$ are sensitive to the accuracy of $D_e$ estimates, we used a range of values ($D; D/2; D/4; D/10$) were $D = \text{the estimated census density across all sacred groves}$ (following Born et al. 2008).
Evaluating patterns of contemporary pollen dispersal:

To investigate contemporary pollen mediated gene flow we applied a paternity analysis on 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 1% 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 LOD 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 metres. This threshold of 500 metres assures for each mother tree that all conspecifics within the same grove are recognised 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: a) Six mother trees as low density with less than five conspecific trees within 500 metres (Average=2.8 trees, median=2.5 trees). b) Eighteen mother trees as high density with six or more conspecific trees within 500 metres (Average = 13.2 trees, Median = 10 trees). c) Two singleton mother trees where 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 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 which 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 \( \frac{d_{\text{obs}}}{d_{\text{ep}}} \) - using the Bayesian approach developed in Klein et al. (2008) with MEMM (v1.1) software (Klein et al. 2011). We modeled the individual fecundities using a gamma distribution including two important covariates (DBH and number of trees within 500 metres). The software uses a Markov Chain randomization. We set the process starting values for the variance of (gamma-) male fecundities \( \sigma^2 \), dispersal distance \( \delta \), shape parameter \( b \), migration rate \( m \) and selfing rate \( s \) as \((\sigma_0, \delta_0, b_0, m_0, s_0) = (2, 100, 1, 0.5, 0.05)\) 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 geographic location of the sampling sites (Figure S1a, Supporting information), suggesting *D. malabaricum* adults form one continuous panmictic population. The juvenile cohorts showed much clearer evidence of genetic structure (Figure 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 ($\sigma$) in adults derived from SPAGEDI (Hardy and Vekemans 2002) ranged from 171 metres to 804 metres 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 x 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 (Figure 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 metres (Figure 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 greater in juvenile pairs within the first six distance classes (less than 200 metres apart), and significantly lower in the
Table 1: Genetic diversity parameters in Dysoxylum malabaricum across life stages

<table>
<thead>
<tr>
<th>Cohorts</th>
<th>N</th>
<th>(N_s)</th>
<th>(H_o)</th>
<th>(H_e)</th>
<th>(R_s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults</td>
<td>235</td>
<td>12</td>
<td>0.628</td>
<td>0.666</td>
<td>10.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(± 1.991)</td>
<td>(± 0.061)</td>
<td>(± 0.066)</td>
<td>(±1.72)</td>
</tr>
<tr>
<td>Saplings</td>
<td>119</td>
<td>10.09</td>
<td>0.606</td>
<td>0.659</td>
<td>10.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(± 1.480)</td>
<td>(± 0.058)</td>
<td>(± 0.071)</td>
<td>(±1.45)</td>
</tr>
<tr>
<td>Seedlings</td>
<td>488</td>
<td>11.36</td>
<td>0.615</td>
<td>0.668</td>
<td>10.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(± 1.739)</td>
<td>(± 0.057)</td>
<td>(± 0.065)</td>
<td>(±1.48)</td>
</tr>
<tr>
<td>Embryos</td>
<td>566</td>
<td>10.82</td>
<td>0.592</td>
<td>0.664</td>
<td>9.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(± 1.536)</td>
<td>(± 0.059)</td>
<td>(± 0.068)</td>
<td>(±1.31)</td>
</tr>
</tbody>
</table>

\(N\): number of samples; \(N_s\): mean number of alleles; \(H_o\): observed heterozygosity; \(H_e\): expected heterozygosity; \(R_s\): Allelic richness based on 117 samples; ± indicates the standard error

Table 2: Estimates of historic gene flow distances (\(\sigma\)) in Dysoxylum malabaricum across life stages

<table>
<thead>
<tr>
<th>Density Estimate</th>
<th>Density (adults per ha)</th>
<th>(\sigma) (m) Adults</th>
<th>(\sigma) (m) Juveniles</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>1.72</td>
<td>171 (±15)</td>
<td>202 (±29)</td>
</tr>
<tr>
<td>D/2</td>
<td>0.86</td>
<td>217 (±29)</td>
<td>241 (±65)</td>
</tr>
<tr>
<td>D/4</td>
<td>0.43</td>
<td>374 (±44)</td>
<td>389 (±131)</td>
</tr>
<tr>
<td>D/10</td>
<td>0.17</td>
<td>804 (±196)</td>
<td>714 (±432)</td>
</tr>
<tr>
<td>(D')</td>
<td>0.01</td>
<td>4741 (±NC)</td>
<td>NC</td>
</tr>
</tbody>
</table>

D: census density within habitat patches; \(D'\): census density across the entire study area; ± indicates the standard error; NC: no convergence

Figure 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).
two distance classes between 300 and 900 metres. The multiclass criterion ($\omega$) shows that the overall correlograms differ significantly ($\omega=73.46$, $P=0.001$).

<table>
<thead>
<tr>
<th>Genetic connectivity of individual trees and habitat patches</th>
</tr>
</thead>
</table>
| 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 metres. Seventy eight per cent (N = 404) of the observed pollination events in progeny arrays occur within groves and do not go beyond 290 metres (Figure 3a). The pollen flow between the groves accounted for 22% (N = 114) of all pollination events. Inter-grove pollination events start at 420 m and reach a maximum of 23.6 km. (Figure 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%). 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. 53 (17%) seedlings were the result of inter-grove pollen dispersal events and one seedling had both parents in two different groves from the one in which it was sampled. The estimates of over all male fecundity variance reveals a high mean $d_{\text{obs}}/d_{\text{ep}}$ 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, Supporting information Figure S3 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 seed with an average pollen dispersal per tree of 100 metres (median = 90 metres). 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.

<table>
<thead>
<tr>
<th>Effect of local tree density on patterns of contemporary pollen dispersal distance and relatedness of parent pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>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 summarised 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 (&lt; 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 isola-</td>
</tr>
</tbody>
</table>
Results | FRAGMENTATION GENETICS OF D. MALABARICUM |

Figure 3: Frequency distribution of pollen dispersal distances in *Dysoxylum malabaricum*.

a) Frequency distribution of within grove pollen dispersal events in 10 metre distances classes from 0 to 300 m, N=404. b) Frequency distribution of inter grove pollen dispersal events in 200 metre distances classes from 0.4 km to 24 km, N=114
Three mother trees with an average pollen dispersal distance of 6.5 km (SE = 1212m, median = 5316m). 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 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 Figure 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 Figure S2, Supporting information).

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

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

SE: Standard error

**Figure 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) forests 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. 2011). 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 and 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. 2010)
2011; Moran and 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).

<table>
<thead>
<tr>
<th>Does local density influence patterns of contemporary pollen dispersal?</th>
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</table>
| 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 of 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.

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<th>Implications of FSGS for inbreeding in highly fragmented landscapes</th>
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| 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 and Hardy 2004), regeneration dynamics after small and large scale disturbance (Premoli and 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 paper, 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. 2011). 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 and Hay 2011). Interestingly, we observe significantly lower relatedness among juvenile pairs within the 300 to 900 metres 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 and Frankham 2003; Leimu *et al.* 2006).

Adopting a precautionary principle for the management of *D. malabaricum*, it would be prudent to maintain grove sizes greater than 6 trees and ensure that distance between groves does not increase. It would be beneficial to assist re-colonisation 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 one kilometre. 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 on-going habitat encroachment and illegal logging. Artificial seed exchange or transplanting of nursery raised seedlings among groves would help to counteract the observed genetic differentiatiation 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 and 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 un-exploited genetic resource. Establishment of native tree seedlings in coffee plantations is hindered by regular weeding. These single adult trees offer an important (un-exploited) 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 and Oldfield 2008). Many of these tropical tree species are likely to exhibit limited seed dispersal (McConkey *et al.* 2011; Kettle *in press*) and significant FSGS. Ensuring local tree densities remain above a critical threshold and that inter-fragment 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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REFERENCES


**Supporting Information**

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

*Numbers in brackets refer to trees in coffee plantations*

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<td>5</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Dm182</td>
<td></td>
<td>14</td>
<td>17</td>
<td>14</td>
<td>17</td>
<td>7</td>
<td>0</td>
<td>9</td>
</tr>
</tbody>
</table>

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)
CHAPTER 2

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

First we conducted a review of the grey literature (master theses from the Forestry College of Ponnampet) for records of *D. malabaricum* in the study area. We then ground-truthed this data with systematic searches of the entire area of each grove, by at least three experienced local field botanists walking repeated transects. In addition we searched all areas in a 200 meter buffer zone around each grove and if possible up to 500 meters in the adjacent coffee plantations. We also consulted local coffee plantation owners for information of *D. malabaricum* on their estates. We also searched all other groves (non-sacred) in local villages where we found trees. Local villagers and temple committee were able to show us individual trees. This approach provided us with a preliminary map of occurrences. Using this map we defined the final study area and using Google earth we were able to target the remainder of our searching to un-explored potential sites of *D. malabaricum*. Finally, after an initial paternity analysis, we conducted an additional search in a 750 meter perimeter of all mother trees with lower assignment. This search revealed an additional six trees of which three trees turned out to be missing fathers.

STRUCTURE analysis of genetic differentiation across life stages.

**Detailed methods**

We applied for each cohort a Bayesian clustering with the software STRUCTURE 2.3.3. In the admixture model for each individual the fraction of ancestry from each cluster is estimated. After trial runs with an assumed number of clusters (K) from 1 to 25 (based upon the maximum number of sample sites with more than 2 individuals) we expected the number of Ks’ to be lower than 10. So for the final results we ran 10 runs for each assumed cluster from 1 to 10 with a burn-in of 30 000 iterations followed by 100 000 Markov Chain Monte Carlo generations. As a formal criterion to determine the most likely number of clusters we applied the ad hoc statistic ΔK developed by Evanno *et al.* (2005). In this approach the ΔK value is plotted against the consecutive number of assumed clusters to detect a maximum rate of change, which indicates the true number of genetically similar clusters.

To compare patterns of genetic clustering among life stages we applied the same analysis for the seedling and sapling samples, with the difference that after trial runs the number of assumed Ks was tested in the final analysis for 1 to 16 clusters.

**Reference**

Figure S1 supporting information: Fraction of ancestry based on Bayesian clustering of the Dysoxylum malabaricum age cohorts with STRUCTURE software for a) the 235 adult samples for 4 assumed clusters, b) the 119 sapling samples for 7 assumed clusters and c) the 488 seedling samples for 8 assumed clusters. The numbers below the bar plots indicate the sampling sites.
Figure S2 supporting information: Boxplot of the number of effective pollen donors (N_{ep}) per mother tree classified according to the local density of conspecific trees within 500 meters.

Figure S3 supporting information: Individual fecundity of all adult trees, showing that most trees contribute little pollen and few trees disproportionately more pollen.
CHAPTER 3

Evaluating realised seed dispersal in a tropical forest tree across a highly fragmented agro-forest landscape using parentage analysis

with J. Ghazoul | G. Ravikanth | R. Uma Shaanker | C.G. Kushalappa | C.J. Kettle |

ABSTRACT

Land conversion and habitat fragmentation are predicted to alter patterns of seed dispersal in many tropical tree species with potentially negative consequences for recruitment and forest community structure. Quantifying realized seed dispersal in tropical trees species between habitat patches at an ecological relevant landscape scale has remained a significant challenge in tropical ecology, despite its importance for understanding forest regeneration. Our aim here was to evaluate the pattern of realized seed dispersal among forest fragments in the threatened canopy timber tree *Dysoxylum malabaricum* (Meliaceae) which depends on the large Malabar Grey hornbills (*Ocyceros griseus*) for seed dispersal. Using a full inventory of all adult *D. malabaricum* trees over an area of 216 km$^2$ in an agro-forest landscape in the Western Ghats and genotypes of 488 seedlings from 19 forest patches we infer patterns of seed dispersal based upon a simple parentage analysis. Contrary to the expectations based upon the size and mobility of the major seed dispersal agent, we found that the vast majority of seed dispersal was less than 150 meters. We demonstrate that in *D. malabaricum* natural colonization of forest patches where this species has been extirpated is extremely unlikely. Our findings suggest that current expectation of seed dispersal among forest patches and natural recruitment of forest tree species based upon assumptions of mobility and size of dispersal agents may be misleading. This has implications for forest dynamics and priority setting of restoration of degraded tropical forests where sites targeted for restoration might be more isolated from seed sources than previously thought.
INTRODUCTION

Seed dispersal is a key factor determining plant species distributions and the structure of most terrestrial ecosystems. Seed dispersal by vertebrates is the dominant mode of dispersal in dry and wet tropical forest often with more than 75% of the tree species producing fleshy fruit (Howe and Smallwood 1982; Willson et al. 1989). The most common animal dispersers are birds which are critical agents for maintaining species composition in tropical forests (Sekercioglu et al. 2004; Sekercioglu 2006) but are often highly threatened by hunting or habitat degradation. This plant animal mutualism might be fundamentally changed or disrupted due to large scale land conversion and disruption of continuous habitats (Uriarte et al. 2011; McConkey et al. 2012) with negative consequences for recruitment of tree species (Cordeiro and Howe 2001) and consequently altered species composition (Moran et al. 2009). Theoretically, the consequences of fragmentation are expected to be less for species which have highly mobile large dispersal agents. On the other hand large dispersers can often be some of the first to be extirpated in degraded and fragmented forests (Cramer et al. 2007).

Our aim is to estimate realised seed dispersal distances in the rare timber species *Dysoxylum malabaricum* (Meliaceae) in a complex agro-forest landscape in Karnataka, South India. Using a full inventory of all the adult trees found in 35 small forest patches over a scale of 216 km², together with an extensive data set of genotyped seedlings we apply a simple parentage analysis to determine the most likely parents. One potential constraint of applying parentage analysis to estimate seed dispersal is that distinguishing between the paternal and maternal parent is ambiguous without the use of maternally inherited chloroplast markers. To overcome this constraint we apply a logical decision based method to selected the maternal parent, based upon our detailed understanding of pollen dispersal in this same landscape (Ismail et al. 2012) and assuming that pollen and seed dispersal distances are entirely independent (see methods for details).

Tree species depend on seed dispersal for re-colonization and range expansion. It is thus imperative to understand seed dispersal patterns if one wants to understand the implications of habitat fragmentation, predict the probability of forest recovery on abandoned agricultural land and to implement efficient ecological restoration strategies of degraded tropical forests (Kettle 2012). *Dysoxylum malabaricum* is a particularly interesting species in this regard as it is not only of conservation concern and economic value but is dispersed by a large bird, the Malabar Grey hornbill (*Ocyceros griseus*) which is known as a relatively strong flier which can cross over unsuitable habitat between fragments (Raman and Mudappa 2003).
We discuss the uncertainties of our seed dispersal estimates in *D. malabaricum* and the conditions under which this approach might be applicable to other tree species. Seed dispersal has been estimated in numerous plant species applying a wide range of non-molecular and molecular methods yet this aspect of plant reproductive ecology remains notoriously difficult, despite technological advances. Seed traps placed at varying distances from a focal maternal plant generally fail to capture the relatively rare but ecologically and evolutionarily important long distance dispersal events (Nathan *et al.* 2003). Indirect molecular approaches using uniparental inherited markers on chloroplast or mitochondrial DNA provide great insights into historic patterns of seed dispersal (see Ennos 1994; Petit *et al.* 2005). But, because at local population scales organelle DNA variation is generally highly conserved such approaches are normally not informative for contemporary patterns of seed dispersal (but see Kamm *et al.* 2009).

Direct methods using maternal matching analyses based on the maternal origin of fruit or seed coat tissue have greatly advanced our understanding of seed dispersal in some temperate forest systems (Godoy and Jordano 2001; Grivet *et al.* 2005). A significant constraint of such direct approaches in tropical systems is the short-lived nature of the seeds and especially the maternally inherited seed coat. Additionally, methods based on matching of the maternal tissue are not possible for estimating realised seed dispersal patterns because the maternal tissue is no longer present in established seedlings. The alternative direct estimates of effective seed dispersal based entirely on parentage analysis usually assume the nearer candidate parent to be the most likely mother, which can lead to underestimates of mean seed dispersal distances (Hamrick and Trapnell 2011) if no other information is considered. In this paper we combine direct estimates of pollen dispersal obtained from seedling samples with the distances of seedlings to the two most likely assigned parents. Based on the assumption that within individual seedlings the seed dispersal and the pollen dispersal distance are independent, the nearer parent is the most plausible maternal candidate.

How far seed move and whether they reach suitable habitat is relevant for understanding recruitment and colonization potential of forest fragments where *D. malabaricum* has been extirpated. Other studies of seed dispersal by vertebrates (e.g. Hardesty *et al.* 2006; Jordano *et al.* 2007; Kamm *et al.* 2009) have observed highly effective long distance seed dispersal. Knowledge of seed dispersal in tropical systems is often based upon observational studies of foraging behaviour and gut passage time of birds. Such estimates may be highly misleading if the realized seed dispersal patterns do not mirror these observations. Such misconceptions could lead to inappropriate forest management or failures in forest restoration. Our study thus endeavors to provide empirical evidence for realized seed dispersal at the landscape scale in one important tree species.
**METHODS**

| Study Area and Study Species

This study is conducted in Kodagu district of the Western Ghats, which contains with 46% a high degree of forest cover (Garcia et al. 2010). In this study we focus on an area of intensively used landscape mosaic dominated by shade coffee plantations and paddy. The study area harbours a high density of small forest patches within a complex agricultural mosaic. Within our study area most of these forest fragments are so called ‘sacred grove’ forests. These ‘sacred groves’ are small forest patches that have been managed and conserved by local communities for worshipping (Chandrakanth et al. 2004). Although the majority of sacred groves are smaller than 2 ha (Kushalappa et al. 2001) and account only for about 2% of the forest cover in the district (Bhagwat et al. 2005a), they are recognized as important repositories of biodiversity (Bhagwat et al. 2005b) within an agricultural matrix.

Within our study area located in the agro-forest landscape of Kodagu we have located all adult *Dysoxylum malabaricum* (Meliaceae) trees within 216 km² (Ismail et al. 2012). This inventory revealed that within our study area out of 235 *D. malabaricum* trees not a single tree was found in the privately owned forest patches and only twelve are found within coffee plantations, highlighting the importance of the sacred groves as habitat for this species. It is noteworthy that during this extensive systematic searching period no seedlings were observed in patches without adult trees. This valuable timber species is classified as endangered under the Indian national threat assessment (Kumar and Ved 2000). *D. malabaricum* produces dull greenish yellow hermaphrodite flowers approximately 7 mm long and 4 mm wide arranged in racemes. The fragrance and the nectar reward suggest insect pollination, but the main pollinators are unknown. The fruit contains 4 carpels with two ovules each of which normally one develops into a seed. When ripe the capsule split along the septa into four sections displaying its bright orange inside and the shiny brown seed (c. 30 x 20 mm) attracting large-gape birds (Ganesh and Davidar 2001). Within our study area the only potential disperser regularly seen foraging on fruiting *D. malabaricum* trees were Malabar Grey hornbills (*Ocyceros griseus*). This hornbill species is known to be more robust to habitat changes than other Indian hornbill species (Raman and Mudappa 2003). The birds consume the entire seed for its brown lipid rich seed coat which is removed before the seed is regurgitated. Seeds which fall directly to the ground with their seed coat still on rot quickly suggesting obligate zoochory (Ismail pers obs 2010). Interestingly the fruiting season of *D. malabaricum* starts just after the fledging of the juvenile Malabar Grey hornbill (Murali 1997; Mudappa 2000). A previous study focusing on genetic connectivity by pollen mediated gene-flow demonstrated that *D. malabaricum* can
attract pollinators over long distances with around 9% of the pollen dispersal events further than 5 km (Ismail et al. 2012).

| Estimating effective seed dispersal: |
| Sampling design and genotyping |
To collect seedling samples we established in 2009 sixty-eight 20 x 20 metre plots across 19 selected forest patches. This sampling design endeavoured to represent the entire range of forest patch areas and number of adults found across our study landscape. Number of reproductive adults per patch ranged from one to 30 adults and area from 0.63 to 14.39 ha. Because seedling densities are much higher beneath fruiting trees and to assure sufficient number of seedlings it was necessary to place 23 of the sampling plots directly beneath fruiting trees. The remaining 45 plots were located randomly across the selected forest patches, with at least one randomly positioned plot in each patch. These random plots assure that we also capture seedlings dispersed further away from fruiting trees. In each plot we collected leave tissue from up to 20 seedlings with less than 50 cm height. We assume that these seedlings generally represent seedlings from the two previous fruiting seasons (2007 and 2008). All seedlings were mapped with a Garmin 60CSx handheld GPS to an accuracy of five metres.

DNA was extracted from the silica gel dried tissue samples using a CTAB extraction method (Sambrook et al. 1989) in the conservation genetic laboratories of the Ashoka Trust for Ecology and Conservation (ATREE). All seedlings 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). Details of PCR conditions are specified in Ismail et al. (2012).

| Parentage analysis |
To investigate seed dispersal we applied a parentage analysis of seedling samples using Cervus 3.0.3 (Marshall et al. 1998). Simulations of parentage 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 1% and 97% of the loci genotyped. The proportion of candidate fathers sampled was estimated to be 95%. The significance threshold for parentage assignments was based upon the 85% and the 95% confidence level of the critical delta LOD score. For each assignment the euclidean distance between the two most likely parents was measured, this unambiguously reveals the realised pollen dispersal
distance. The observed clear trend of increasing pollen dispersal distances with the reduction of the confidence levels indicates that these long distance events are more likely to contain erroneous assignments. To ensure a high confidence in assignments over longer distances where the potential for un-sampled parents is greater we only included the assigned seedlings beyond two kilometres if they were assigned at the 95% confidence level.

Logical decision based maternal tree assignment

Because it is impossible to know with certainty which tree is the mother and which is the father based upon the offspring genotype, we adopted the following logical decision rules to infer the maternal tree and estimate seed dispersal distance. When both the assigned parents were found within the same forest patch as the seedling or in a different patch to the seedling we calculate the minimal and maximal seed dispersal distance based upon a) the mother being the nearest parent to the seedling (minimal distance) and b) the mother being the most distant parent from the seedling (maximal distance) following Finger et al. (2011).

When a seedling was assigned to one parent in the same forest patch and to another parent in a different patch we infer the nearest tree to the seedling is the maternal tree. This decision is based upon the assumption that the pollen and seed dispersal distances are independent. We can reject maternal offspring relationships where pollen and seed dispersal distances are strongly correlated and consequently assign the alternative parents as most likely seed source (see supporting information Appendix S1 for more detailed justification). A second line of evidence to reject one parent as seed source is based on the expected frequency of inter-patch pollen dispersal events. Because seeds which are dispersed from one forest patch to another are expected to represent a random sample such seeds should represent a similar ratio of inter-patch pollen dispersal like randomly sampled seeds. We therefore additionally tested the probability of the observed inter-patch pollen dispersal frequency with a binominal test using the frequency of 22% of inter-patch pollen dispersal events as expected frequency (see supporting information Appendix S1 for more details). This expected frequency is obtained from the paternity analysis of seed sampled directly from mother trees in a former study (Ismail et al. 2012). Using this information we were able to select the most likely maternal tree based on the probability of inter-patch pollen versus seed dispersal.
RESULTS

| Parentage assignment |
Of the 488 genotyped seedling samples assigned to parents using CERVUS all could be assigned at the 85% confidence level. Because this confidence level certainly includes some erroneous assignments and because the potential for assignments to un-sampled parents is greater over longer distances, parent pairs greater than 2 kilometres apart were only included when they could be assigned at the 95% confidence level. This reduces the total number of seedling parent pair combinations used to infer seed dispersal to 77% of our total sample (N=376). Of these assigned seedlings 83% (N=311) had both candidate parents within the same forest patch as the seedlings reflecting within patch seed dispersal. The range of distances between the seedling and either parent ranged from 1 m to 407 m. Assuming that the nearest assigned parent was the mother (minimal distance) average seed dispersal distance was 38 m. Assuming the most distant parent was the mother (maximal distance) the average seed dispersal distance is 95 m (see Table 1 for a summary).

Table 1: Summary of the minimum and maximum seed dispersal distance estimates and the pollen dispersal estimates of Dysoxylum malabaricum based on parentage analysis

<table>
<thead>
<tr>
<th>Candidate parent location relative to seedling forest patch</th>
<th>N</th>
<th>%</th>
<th>Minimum seed dispersal estimate</th>
<th>Maximum seed dispersal estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean (median) Range</td>
<td>Mean (median) Range</td>
</tr>
<tr>
<td>Both parents in same patch</td>
<td>311</td>
<td>83%</td>
<td>38 m (17 m) 1 m – 327 m</td>
<td>95 m (79 m) 6 m – 407 m</td>
</tr>
<tr>
<td>One parent in another patch</td>
<td>64</td>
<td>17%</td>
<td>55 m (38 m) 1 m – 212 m</td>
<td>55 m (38 m) 1 m – 212 m</td>
</tr>
<tr>
<td>Both parents in another patch</td>
<td>0</td>
<td>0%</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Both parents in two different patches</td>
<td>1</td>
<td>0.3%</td>
<td>2 521 m na</td>
<td>10 909 m na</td>
</tr>
<tr>
<td>Total assigned Parents</td>
<td>376</td>
<td>100%</td>
<td>47 m (20 m) 1 m – 2521 m</td>
<td>95 m (77 m) 1 m – 10 909 m</td>
</tr>
</tbody>
</table>

Only 17% (N=64) of the seedlings assigned had one candidate parent in a different forest patch to the seedling which could represent inter-patch seed dispersal. However, because in all these cases assuming the most distant parent is the mother violates our assumption of independences between pollen and seed dispersal distances (see supporting information Appendix S1 for detailed justification) we can confidently assign the mothers as the trees in the same patch as the seedling, which in all cases was the nearest assigned parent. Here the seed dispersal distance ranged from 1 m – 212 m with an average of 55 metres. Only one seedling, were both
candidate parents are located in different forest patches to the seedling, demonstrates unambiguously inter-patch seed dispersal. In this case the distance from the seedling to the nearer candidate parent is 2.5 km and to the further 10.9 km. In this case we cannot unambiguously discriminate between maternal and paternal tree and therefore include in our overall estimates the nearer distance in the minimum estimate and the further distance in the maximum estimate (see Figure 1).

**Figure 1: Frequency of seed dispersal distances in Dysoxylum malabaricum, N=376, the light grey bars refer to the minimum seed dispersal distances, the dark grey bars refer to the maximum seed dispersal distance, the distance class “More” refers to one seed dispersal event for which it is unclear if the dispersal distance was 2.5 km or 10.9 km**
DISCUSSION

Investigating contemporary realized seed dispersal using parentage analysis we could assign 77% (N=376) of the genotyped seedlings to the two most likely parents with a high confidence. The majority of parent pairs were found in the same forest patch as the seedlings and in only one case the two most likely parents were in two different patches than the seedling. Of the 65 seedlings where at least one parent was assigned from a different forest patch to the seedling we could confidently reject the further parent as the maternal tree. Based upon these patterns of seed dispersal we conclude that seed dispersal is limited in these forests despite the potential for the dispersal vector to transport seed over long distances. Below we discuss the evidence for limited seed dispersal, the more general implications for patterns of dispersal by large birds in fragmented landscapes, and the significance of our findings for tropical forest recovery and restoration in agro-forest landscapes.

Evidence for limited seed dispersal

Our results support the view that most seeds are dispersed only relatively short distances from mother trees. Indeed the vast majority (83%) of parent pairs were assigned within the same forest patch as the seedling. Even if we could not unambiguously assign the maternal parent here the differences between the minimum and the maximum seed dispersal distances we estimate are minor. At the minimum 98% (N=306) and at the maximum 94% (N=293) of the assigned seed are dispersed less than 200 metres. In the 65 seedlings where one parent was assigned in a different forest patch to the seedling, inter-patch pollen dispersal provides a far more plausible explanation of the parental relationships (see supporting information Appendix S1). Thus we reject the unlikely scenario here that putative mother was in a different patch to the seedling and that the seed by chance was always returned to the same forest fragment as the pollen donor. Only one long distance seed dispersal event of between 2.5 km or 10.9 km (depending on the assignment of the mother) was observed, which suggests effective seed dispersal over these larger scales is very rare but possible. Based on our seed dispersal estimates it is likely that seeds are regularly dispersed into the surrounding shade coffee plantations which could increase the actual dispersal distance, but because seedling establishment is suppressed by regular weeding this will not result in realized dispersal.

One caveat in our analysis may be that we exclude 23% (N = 112) of our seedlings because these assigned parent pairs were greater than 2 kilometres apart. We cannot entirely exclude that these include some long distance seed dispersal events, and thus bias our analysis to short distance dispersal. However, it is noteworthy that in all these seedlings, the parents
pairs assigned at the 85% confidence level were all in two distinct forest patches different to the seedling and represent parent pair distances which are incompatible with our previous direct and unambiguous estimates of pollen dispersal. Alternative explanations for these seedling parent relationships thus include, erroneous assignments, pollen inflow from outside our study area, unsampled maternal trees, or even maternal trees that were logged out before our sampling started.

The here used approach to directly estimate realised seed dispersal based on parentage analysis can be only applied in fragmented plant populations where pollen flow clearly exceeds seed dispersal and where between-patch distance exceeds within-patch distance. As tree often species show much greater pollen dispersal than seed dispersal (Petit et al. 2005) and many tropical tree species have limited seed dispersal and high clustering of conspecifics (Seidler and Plotkin 2006) there might by plenty fertile ground for investigation. However, this may be less feasible in systems where seed dispersal is expected to exceed pollen dispersal for example species with wind dispersed seeds and pollination by highly immobile pollinators, and have a large number of candidate parents within a population.

| Are large bird dispersers always effective dispersal agents? |

The distinct challenges associated with tracking seed dispersal in the field are undisputable. Numerous alternative methods ranging from field observations, re-locating marked seed or seed traps have been applied (for method details see Bullock et al. 2006 and references therein). In animal dispersed species seed dispersal is often inferred form general movement patterns in combination with feeding behaviour and gut passage times (Bullock et al. 2006). Indeed, all studies we are aware of investigating dispersal by hornbills rely on such observational methods and all suggest that hornbills are both important and effective seed dispersal agents in tropical forest systems (Whitney et al. 1998; Holbrook and Smith 2000; Holbrook et al. 2002; Kitamura et al. 2004; Kitamura et al. 2006). Our results suggest that the ecologically relevant, realised seed dispersal by hornbills may be far shorter than predicted based upon such field observations. Field observations of seed dispersal by hornbills often focus on the breeding season by observing hornbills or by collecting seed at the nesting sites although foraging range of hornbills can change dramatically over the year (Poonsawad and Tsuji 1994; Datta and Rawat 2003). In the case of D. malabaricum the fruiting season is just after the fledging of the juvenile Malabar Grey Hornbills where small family flocks can be regularly observed foraging (pers. obs. 2009 and 2010). It seems plausible that the juveniles in these flocks might reduce the hornbills foraging range especially when food resources are clustered in forest fragments. Nathan et al. (2008) suggest that larger animals with larger home ranges
and higher travel velocity as well as longer seed retention time promote long distance seed dispersal. Indeed, there are examples of seed dispersal studies applying molecular techniques which are in line with this generalization (e.g. Hardesty et al. 2006; Jordano et al. 2007; Kamm et al. 2009). Therefore our finding of very localized seed dispersal appears to provide an exception to this generalization, but it is not the only exception as shown by Grivet et al. (2005) by applying maternal matching to pericarp tissue of bird dispersed acorns. However, our study demonstrates that the possibility of very localized seed dispersal should be taken into account when estimating seed dispersal potential based on the size or the general movement patterns of the animal vector.

| Implications for forest dynamics and restoration of degraded sites |

Our estimates of effective seed dispersal demonstrate that seed of *D. malabaricum* are dispersed almost exclusively within forest fragments. Migration among fragments and re-colonization of forest patches where *D. malabaricum* has been logged thus appears to be highly unlikely. The minimum distance between any *D. malabaricum* tree in our study area to an adjacent forest patch is 370 metres, further than 99% of our estimated seed dispersal distances. Even with the potential for very rare cases of successful migration into forest patches where the species is not present, natural colonization will be extremely slow. Considering the longevity of trees such rare events might contribute to the persistence of *D. malabaricum* population. Evaluating the effect of such rare events for the population dynamics at the landscape scale is extremely difficult with long-lived species and beyond the scope of this study. Our results do suggest that dispersal of *D. malabaricum* seeds into adjacent coffee plantations is extremely likely. Thus the current activities of farmers to weed out native trees from coffee plantations must represent a considerable waste of valuable native tree genetic resources.

Management intervention through artificial seed dispersal or transplanting of nursery raised seedlings to colonise forest patches would certainly help to maintain connectivity and the persistence of this species. This could help to mitigate the negative genetic consequences of fragmentation already observed in this species (Ismail et al. 2012). To avoid negative fitness consequences within the remaining forest patches human mediated gene flow among forest patches by seed or seedlings should therefore also consider forest patches where adult trees already occur, especially forest patches where there are only few trees left.

Although many studies have investigated the interactions between tropical tree species and their dispersers, very few have actually quantified realised seed dispersal (Kettle 2012). The limited seed dispersal observed in our study has significant implications for fine-scale spatial
genetic structure (FSGS), a phenomenon common in many important tropical tree species (Hardy et al. 2006; Born et al. 2008; Harata et al. 2011; Kettle et al. 2011) that can increase under habitat disturbance (Premoli and Kitzberger 2005). If tree species experience more restricted seed dispersal as a consequence of habitat fragmentation (Cramer et al. 2007; McConkey et al. 2012) then FSGS may become more intense in modified agro forest landscapes. Restoration efforts such as direct seeding may be essential to mitigate these processes and ensure the persistence of diverse forest communities which support forest biodiversity.

<table>
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<td>We would like to thank the diligent field assistants Navin H. Kumar, Monappa C.S., Chengappa S.K., Range Gowda and the late Umesh. The laborious work of single DNA extraction tidily conducted by Sandeep Sen and Shruthi Jayappa is esteemed a lot. Fragment analysis was conducted at the Genetic Diversity Centre (GDC) of ETH Zürich. This research was funded by ETH Zürich under the grant number ETH-22 08-2.</td>
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REFERENCES


References | SEED DISPERSAL D. MALABARICUM |


**SUPPORTING INFORMATION**

![Figure S1: Plot of pollen dispersal distance against two scenarios of seed dispersal represented by the distance of the seedling to further candidate parent (left graph) and the nearer candidate parent (right graph) based on parentage analysis in *Dysoxylum malabaricum*. Based on a linear regression (dashed line) we reject the scenario displayed in the left graph because it violates the assumption of independent pollen and seed dispersal.](image)

| Appendix S1: Justification for nearest mother assignment in inter-patch parent pairs |

In the 65 cases where one or both candidate parents are outside the forest patch of seedling collection, the regression of the distances between the two most likely parents and the distances between the seedlings to the more distant parent shows an unexpected and extremely strong linear correlation ($p < 0.0001$, adjusted $R^2 = 0.993$) whereas the alternative that the nearer parent is the seed source shows no significant correlation ($p = 0.19; R^2 = 0.012$) (See Figure S1 in the supplementary information). This is in line with the assumption that pollen and seed dispersal are independent and indicates a clumped seedling distribution around one of the parent trees, which we assume therefore is the maternal tree.
To confirm this finding we additionally test the expectation that the 65 events of potential seed dispersal represent a random pollen composition. To test whether this scenario is possible we apply the frequency of 22% inter-patch pollen dispersal events as hypothesized expected probability of a binomial distribution. This frequency was observed in the paternity analysis conducted with the embryo samples in a previous study (Ismail et al. 2012). A one tailed binomial test computed in R (R Development Core Team 2011) revealed that on a 95% confidence level the observed “success” rate of 65 out of 65 pollination events from outside the forest patch is significantly greater than the expected probability of 22% (p < 0.0001). Consequently we can reject that all these 65 seedlings derive from inter-patch seed dispersal events. For the 64 seedlings which have only one parent in another forest patch we therefore interpret that this parent is the true pollen donor.

References
CHAPTER 4

Beyond fragmentation: Ecological and genetic implications of fragmentation and degradation for recruitment of a rare tree in a tropical agro-forest landscape.

with J. Ghazoul | G. Ravikanth | R. Uma Shaanker | C.G. Kushalappa | C.J. Kettle |

ABSTRACT

Tropical forest fragmentation and degradation seriously threaten global biodiversity and ecosystem services. Our understanding of the implications of both these processes for tropical tree recruitment remains limited. Such knowledge is essential for policies and management strategies that aim to mitigate negative effects and sustain diverse and functioning tropical agro-forest landscapes.

Combining ecological and demographic data with a molecular assessment of inbreeding we evaluate recruitment of the threatened timber species *Dysoxylum malabaricum* (Meliaceae) in highly fragmented forest patches within a complex agro-forest landscape of the Western Ghats biodiversity hot spot, South India. Quantification of habitat degradation and recruitment from 17 forest patches suggest that increased canopy openness and the abundance of pioneer species leads to a general decline in the suitability of forest patches for the recruitment of *D. malabaricum*. The evaluation of inbreeding in 297 nursery reared seedlings using eleven microsatellite loci shows that elevated mating between related individuals leads directly to reduced seedling vigor.

We conclude that elevated inbreeding coupled with increased canopy openness and a less suitable habitat for seedling establishment due to both fragmentation and degradation limit the recruitment of the important timber tree. Management strategies which enhance canopy cover and ensure local densities of adult trees in agro-forest mosaics will be required to ensure *D. malabaricum* persists in these landscapes. Our findings highlight the potential for these incipient processes to threaten the viability of populations of many important and rare tropical tree species in human dominated landscapes.
INTRODUCTION

Tropical forests are globally important centers of biological diversity and provide crucial ecosystem services including important terrestrial carbon stores (Naidoo et al. 2008). Most tropical forests are not in protected areas (Brooks et al. 2004) and remain mainly as logged, degraded or secondary forests (Wright and Muller-Landau 2006). The ecological integrity of these biomes is threatened by agricultural expansion and intensification which has resulted in forest fragmentation and degradation (Bradshaw et al. 2009). Synergistic effects of such co-occurring stressors are thought to be major drivers of biodiversity loss (Laurance and Useche 2009). However, very little empirical research has been conducted to evaluate potential interactions between degradation and fragmentation for the main components of a tropical forest, its trees.

Habitat fragmentation can be defined as a landscape-scale process of habitat loss resulting in the disruption of habitat continuity (Fahrig 2003). Fragmentation affects the reproductive ecology of many plant species by disrupting pollination and seed dispersal processes (Aizen and Feinsinger 1994). Individual plants belonging to small isolated populations often receive fewer pollinator (Cunningham 2000) and seed disperser visits (Rodriguez-Cabal et al. 2007) which directly reduces reproductive output. Seedling establishment and seedling survival might also be reduced by edge effects which cause drier conditions (Uriarte et al. 2010), increased litter fall which smothers seedlings (Scariot 2000), and alien species invasion (Hobbs 2001). Recruitment of shade tolerant plants is thought to be especially sensitive to fragmentation effects because of competition with faster growing light-demanding plants that perform better under more open canopies (Benitez-Malvido and Martínez-Ramos 2003). Additionally, the loss of animal species due to forest degradation further affects recruitment of animal dispersed plants (Moran et al. 2009). Forest fragmentation can thus cause shifts in species compositions where slow growing climax tree species are replaced by early successional species (Laurance et al. 2006). These changes might have implications for both the overall diversity of remnant forest and the ecosystem services they provide.

Forest fragmentation is often associated with anthropogenic degradation of remnant forest by logging, grazing, hunting and invasive exotic species (Tabarelli et al. 2004) among others. These processes are predicted to further affect tree recruitment beyond the effects of reduced population sizes (Brook et al. 2008). From a conservation genetic perspective it is expected that fragmentation erodes genetic variability and increases inter-population genetic divergence due to increased genetic drift and inbreeding, as well as reducing gene flow.

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among increasingly isolated subpopulations (Aguilar et al. 2008). Studies investigating genetic consequences of fragmentation in woody species reveal that fragmentation leads to elevated inbreeding (e.g. Kettle et al. 2007; Dick et al. 2008) and reduced fitness of the progeny (Cascante et al. 2002; Breed et al. 2012a). A possible underlying process is that pollinators predominantly forage among near neighbors, which increases bi-parental inbreeding due to increased mating among few related individuals (Breed et al. 2012b; Ismail et al. 2012).

Our objective was to explore the ecological and genetic processes which influence tree recruitment in an increasingly human-dominated landscape. We investigate the effects of genetic and ecological stressors on the growth and recruitment of *Dysoxylum malabaricum*, a highly prized and rare timber species which is found in a highly fragmented agro-forest landscape in Southern India. A previous study demonstrated that despite extensive pollen dispersal *D. malabaricum* experiences elevated inbreeding and reduced pollen diversity when local populations become highly fragmented (Ismail et al. 2012). The aim of this study is to empirically investigate the processes compromising recruitment in this species and, specifically, to examine the relative ecological and genetic stressors. We ask the following questions: A) Does mating between related individuals reduce seedling performance in *D. malabaricum*? B) Does ecological patch degradation influence local seedling density in *D. malabaricum*? To address these questions we conducted ecological surveys of seedling recruitment across 85 plots in 17 forest patches, from which we gathered both genetic and ecological data, and applied multiple linear regression (MLR) models to determine the most important determinants of seedling density. In addition, we established a nursery experiment of genotyped seedlings to investigate if inbreeding affects seedling growth. Based upon field observations and experimental results we explore the relative importance of spatial isolation, habitat patch suitability and genetic processes for the recruitment of *D. malabaricum*.

Tropical agro-ecosystems provide heterogeneous mosaic habitats increasingly important for biodiversity conservation (Perfecto and Vandermeer 2008). Our research highlights that the genetic consequences of fragmentation and the ecological consequences of habitat degradation need to be considered in unison if important canopy tree species such as *D. malabaricum* are to persist in these novel landscapes. We discuss the wider relevance of our findings for other threatened tropical tree species which are progressively reduced to habitat fragments in human dominated landscapes.
CHAPTER 4

METHODS

| Study area |
The study area focuses on an intensively used agricultural landscape encompassing 216 km² at a distance of at least 2 km from any continuous forest in Kodagu district, within the Western Ghats biodiversity hotspot in South India. This district is a major coffee-growing region in India where coffee is grown predominantly under native shade trees (Garcia et al. 2010). Beside a high degree of 46% of forest cover (Garcia et al. 2010) the district is also known for the high density of small native forest patches within an agricultural matrix consisting mainly of native shade coffee plantations and paddy (Kushalappa and Bhagwat 2001). Although these forest patches within the agricultural matrix contribute marginally to the overall forest cover of the region (Bhagwat et al. 2005a), they are important repositories of biodiversity (Bhagwat et al. 2005b). These forest patches are subject to disturbance through intensified resource extraction of fuel wood, small poles and non-timber forest products (like fruits, honey, medicinal plants) by the local community (Garcia C.A. and Pascal J.P. 2006), but also illegal timber and wood extraction and the encroachment of forest patches by coffee plantations (Chandrananth et al. 2004). Based on a detailed pollen flow study within the landscape of Kodagu we have evidence that we have located virtually all adult *D. malabaricum* trees within the 216 km² area (Ismail et al. 2012).

| Study species |
*Dysoxylum malabaricum* (Meliaceae) has been extensively logged (Anonymous 1952), and demand for *D. malabaricum* timber remains high with round wood logs fetching up to 620 US $ per cubic metre at local timber auctions in Kodagu (Ismail pers. obs. 2008). The species is not assessed under the IUCN Red List but is classified as endangered under the Indian national threat assessment (Kumar and Ved 2000). It produces hermaphrodite flowers with a nectar reward suggesting insect pollination, but the main pollinators are unknown. The fruit contains four carpels with two ovules each of which normally one develops into a seed. The ripe fruit split along the septa displaying the shiny brown seed (c. 30 x 20 mm) which are food for large-gape birds (Ganesh and Davidar 2001). During two consecutive fruiting seasons the only potential disperser regularly seen foraging on the fruiting *D. malabaricum* trees were Malabar Grey hornbills *Ocyceros griseus* Latham. The birds ingest the entire seed and remove the brown lipid rich seed coat before regurgitation. If the seed are not consumed by birds they fall to the forest floor and rot quickly suggesting obligate zoochory (Ismail pers. obs. 2010).
Within our study area *D. malabaricum* is predominantly found in forest patches: of the 235 adult trees recorded, 223 trees were found in 35 forest patches. The remaining twelve trees were found in coffee plantations (of which nine were found within 300 meters of a forest patch).

**Evaluation of seedling performance and inbreeding under nursery conditions**

**Controlled seedling growth**

Recruitment may be influenced by genetic processes such as inbreeding depression. We evaluate the performance of genotyped seedlings of known provenance in a controlled nursery experiment over 21 months. 617 seeds were sampled near to 37 fruiting trees (97% of seed were found within 10 meters of a fruiting tree) across 16 of the sites within our study area (see Table S1 in Supporting Information for details). These seeds were planted in 1 liter polyethylene bags into a mixture of 1 part red soil, 2 parts cow dung and 2 parts river sand during July and August 2008. A total of 363 seeds (61%) germinated, at least 119 seeds (20%) were predated by a maggot infestation of a tephritid fly (*Dacus sp*). Because of this initial exogenous loss we did not continue assessment of germination rates as a performance variable attributable to genetic factors. The subsequent seedlings (n= 363) were kept in a shade house (50% shade cloth) and watered daily. The seedlings were rotated monthly within the shade house to prevent any local effects, such as daylight orientation and neighbor competition. To reduce mortality due to pests we applied insecticide and fungicide uniformly across the plants. Plants were repotted after 1 year into 5 liter polyethylene bags. Seedling growth was evaluated on a monthly basis by measuring the total stem length from soil to top of the apical meristem, with an accuracy of 0.5 cm.

**Evaluation of seedling inbreeding**

For each of the genotyped nursery raised seedlings we first determined the two most likely parents based upon maximum likelihood analysis using previously published genotype data on all the adults in our study area (Ismail *et al.* 2012) and the genotypes of each seedling at the same eleven loci (see Appendix S1 in Supporting Information for details on genotyping and genetic analysis).

To investigate if inbreeding and mating between related individuals reduces fitness of the seedlings we first applied a parentage analysis with the delta maximum-likelihood approach implemented in CERVUS (Marshall *et al.* 1998) to detect the two most likely parents (see Appendix S1 for details of the parameter settings). After determining the two most likely parents at the 90% confidence level we calculated the kinship coefficient (Loiselle *et al.* 1995) of
the parent pairs as one measure of inbreeding. We also calculated the individual inbreeding coefficient (Ritland 1996) implemented in the program SPAGEDI 1.3a (Hardy and Vekemans 2002) and based on the seedling genotypes alone. The individual inbreeding coefficient $\hat{\rho}$ is calculated as:

$$\hat{\rho} = \frac{\sum_{i,l} (S_{il} - P_{il}^2) / P_{il}}{\sum_{l} (n_l - 1)}$$

where $P_{il}$ is the estimated frequency of the $i_{th}$ allele at the $l_{th}$ locus, $S$ is an indicator variable which is one if the individual is homozygous for the $i_{th}$ allele at the $l_{th}$ locus and zero otherwise, and $n_l$ is the number of alleles at the $l_{th}$ locus.

Ideally pedigree based indices of inbreeding (see Pemberton 2004 and references therein) should be used to evaluate inbreeding depression. However, this is impossible to apply in natural populations of long lived highly fecund tropical trees. We apply the two different methods to estimates inbreeding; individual inbreeding coefficient (Ritland 1996) and ‘kinship’ of parent pairs (Loiselle et al. 1995), as an alternative to the pedigree approach or heterozygosity fitness correlations which are sensitive to the biases associated with using only few neutral loci (Szulkin et al. 2010).

<table>
<thead>
<tr>
<th>Characterizing fragmentation of <em>D. malabaricum</em> habitat</th>
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| We characterize level of fragmentation of the *D. malabaricum* forest patches focusing on 17 selected forest patches within agricultural matrix based upon the number of trees within 500 metres of any given tree within a patch. This characterization of fragmentation combines spatial and genetic isolation (Ismail et al. 2012) with the local density of the focal species. The threshold of 500 metres assures that all conspecifics within the same forest patch are recognised and accounts for the rare cases for higher local densities if more conspecifics are in nearby groves. The patches were selected based upon their level of fragmentation, to ensure replication of both size and isolation. We then categorized fragments into highly fragmented (with fewer than 6 trees within 500 metres) or less fragmented (more than six trees within 500 m) following Ismail et al. (2012). This parameter reflects the local abundance of adult individuals of *D. malabaricum* and the distance to the neighboring forest patches and abundance of adult individuals within those neighboring patches. Only five of the 17 forest patches had another forest patch within 500 metres and the distance between any two trees within a patch never exceeded 450 metres. The threshold distance of 500 metre radius is based upon detailed genetic analysis of pollen and seed dispersal distances, which shows that dispersal rarely exceeds 500 m (Ismail et al. 2012).
Evaluation of habitat degradation and recruitment of *D. malabaricum*

Sample plots

To quantify habitat degradation and the recruitment of *D. malabaricum* under natural conditions, we established five random plots in each of the 17 selected forest patches (85 random plots) in 2010. For each patch we selected plots randomly using north orientated maps with an overlaid grid. Each grid cell was assigned a number, line by line from the top left to bottom right. We then used a random number generator in the program Excel™ (Microsoft Corporation) to select five random plots for each patch. The lower left-hand corner of the plot was defined by these coordinates. In case of overlap with other plots or when more than 10% of the plot was outside the forest patch the plot was turned 90° clockwise until the entire plot lay within the patch. Plots were 14 m x 14 m except in the smallest forest patch where we had to reduce plot size to 10 m x 10 m. All plot based measures were standardized to 100 m². To quantify recruitment of *D. malabaricum* we recorded the number of seedlings (<50 cm height), number of saplings (>50 cm, < 150 cm) and poles (> 150 cm and DBH <5 cm) in each plot.

Ecological parameters to quantify habitat degradation

Within each plot we recorded seven parameters which reflect the forest structure and species composition as indicators for habitat degradation. These included three variables indicative of low habitat quality: 1) Canopy openness measured with a densiometer (model A) (Lemmon 1956). 2) The number of coffee seedlings (<150 cm height), because coffee *Coffea canephora* (a common and important exotic crop species) is naturalizing within forest patches of our study area (Ismail pers. obs. 2009). 3) Juvenile (>0.25m; <2m) abundance of the pioneer tree species *Clerodendrum viscosum* a light demanding species which dominates degraded forest. Four variables were selected as indicators of good habitat quality: 4) the number of adult *D. malabaricum* trees within a patch; 5) the total area of each forest patch (by mapping the forest patch with a GPS handheld (60CSx, Garmin, USA) on an accuracy of five meters). 6) The proportion of the forest patch border by shade coffee plantations. We chose this variable as shade coffee plantation of the region are predominately grown under dense native shade providing a more forest like matrix than the other land uses in the study area. This might buffer edge effects more effectively. 7) In addition we quantified the number of arboreal termite nests attached to branches and trees within each plot. Termites have been shown to be good indicators of forest disturbances (Eggleton *et al.* 1996; Jones *et al.* 2003; Alves *et al.* 2011). Using all these variables as indicators of habitat quality and *D. malabaricum* seedling density as an indicator of recruitment we test the hypothesis that recruitment is reduced in degraded forest patches.
<table>
<thead>
<tr>
<th>Statistical analysis</th>
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<tr>
<td>Evaluation of inbreeding under nursery conditions</td>
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</table>
Because the effect of local tree density in a forest patch has already been demonstrated to influence patterns of inbreeding in *D. malabaricum* (Ismail et al 2012) we classified the nursery seedlings into those collected from forest patches of low density (LD) and those from high density patches (HD). We tested for significant differences of average seedling size, average individual inbreeding coefficient and average kinship coefficient between LD seedlings and HD seedlings using non parametric Wilcoxon rank sum test. See Appendix S2 for details of this classification and of the analysis.

<table>
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<tr>
<th>Evaluating ecological degradation and recruitment</th>
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</table>
All statistical analysis were performed with R 2.13.1 (R Development Core Team 2011). To account for the nestedness of the study design the measurements of the five plots per forest patch were averaged before analysis. This resulted in 17 observations. To investigate reproductive success of *D. malabaricum* in the wild, MLRs were performed with seedling densities as the response variable and as explanatory variables, the variables on habitat degradation. On this initial model we applied model selection with the step function implemented in R to exclude irrelevant variables and to improve the model (see Appendix S2 for more details).
RESULTS

| Evaluation of inbreeding and seedling performance |
Of the 363 seeds which germinated 297 seedlings survived for 21 months. Using a parentage analysis we were able to assign 99% of seedlings at the 90% confidence level to the two most likely parents (293 individuals). In 74% (217 individuals) of nursery seedlings both parental trees were assigned within the same forest patch the seeds were collected from. In 96% (280 individuals) of seedlings at least one parent was found within the same patch as the seed was collected. Of these seedlings the distance between the seed and nearest assigned parent never exceeded 261 meters. Only 13 seeds (4%) were found to be sired by two parents both in another forest patch. Here the distance between the location where the seed was collected and the nearest parent always exceeded 500 meters. In 65% (191 individuals) of the cases the tree under which the seed was collected was one of the putative parents.

Comparing seedlings originating from HD forest patches vs LD forest, the median ranks of the seedling size after 21 months are significantly higher (p-value = 0.00003), individual inbreeding coefficients and kinships of parent pairs significantly lower (p-value = 0.001 and p-value = 0.0001 respectively) in seedlings from HD forest patches (Figure 1). Individual inbreeding coefficient was significantly negatively correlated with seedling size (Pearson’s product moment correlation coefficient = -0.139, t = -2.36, df = 281, p-value = 0.02). In contrast, we observed no significant correlation between kinship of parent pairs and size of the seedlings (t = -1.32, df = 271, p-value = 0.19).

| Population structure |
Although seedlings and saplings are present in nearly all surveyed forest patches, (396 and 68 respectively) pole stage trees (> 1.5 m height and < 5 cm DBH) were extremely rare. Only 11 poles were detected in total in six of 17 surveyed patches. Seedling and sapling densities vary greatly among sites. Seedling densities (averaged per site) range from 0.4 to 50 individuals per 100 m² and sapling densities from 0 to 12 individuals per 100 m². Pole stage trees were found at extremely low densities from 0.1 to 0.4 individuals per 100 m². Plotting size class frequency distributions of all recorded reproductive (> 5 cm DBH) D. malabaricum trees across the entire 216 km² area shows an inverted ‘U’ shape distribution (Figure 2). There was a noticeable absence of the smallest size classes (< 20 cm DBH) with only one tree with a DBH of 8.5 cm and no trees in the 10 – 20 cm DBH class. There is a constant decline in the frequency of individuals from 60 cm DBH downward.
Figure 1: Effect of high density (HD) and low density (LD) of adult Dysoxylum malabaricum trees on A) seedling size after 21 months of growth, B) individual inbreeding coefficient and C) pairwise parental kinship coefficients (Loiselle et al. 1995) of nursery raised D. malabaricum seedlings. Significant differences are based on Wilcoxon rank sum test; * p<0.05, ** p<0.01, *** p<0.001.

Figure 2: Histogram of diameter at breast height (DBH) of all the 235 enumerated adult Dysoxylum malabaricum trees within the 216 km² study area.
Evaluation of habitat degradation and recruitment of *D. malabaricum*

Indicators of habitat degradation were used as explanatory variables of a MLR to investigate their effect on seedling densities. In the model selection the number of adult *D. malabaricum* trees, the area of the forest patch and the density of coffee seedlings, did not improve the regression model and were excluded from the subsequent model. Within the final reduced model (Table 1) the percentage of closed canopy (t=3.67, p=0.0032), the density of *C. viscosum* juveniles (t = -5.05, p=0.0003) and the density of termite nests (t = 7.21, p=0.00001) are significantly correlated with seedling density at the 5% level. The proportion of the grove bordered by coffee plantations improved the model but is not significantly positively correlated with seedling density (t = 1.54, p = 0.149). The adjusted r squared of the final model is 0.81. Restricting the MLR only to the three significant variables results in an adjusted r squared of 0.79.

*Table 1: Summary of the multiple linear regression after model selection used to fit *Dysoxylum malabaricum* seedling densities under natural conditions. Significance level codes: 0 ‘***’; 0.001 ‘**’; 0.01 ‘*’; 0.05 ‘.’; 0.1 ‘ ’*

|                      | Estimate | Std. Error | t value | Pr>|t|]   |
|----------------------|----------|------------|---------|-------|
| (Intercept)          | -35.007  | 9.796      | -3.573  | 0.0038 ** |
| % of closed canopy   | 0.375    | 0.102      | 3.671   | 0.0032 ** |
| *C. viscosum* juveniles | -0.286  | 0.057      | -5.053  | 0.0003 *** |
| Termite nests        | 3.918    | 0.543      | 7.213   | 0      *** |
| % of border with coffee | 0.008    | 0.005      | 1.542   | 0.1491 |

Results
DISCUSSION

In this study we combine genetic data of nursery grown seedlings in *Dysoxylum malabaricum* with detailed ecological data on demography and recruitment. Our results indicate that fragmentation causes reduced seedling vigour through genetic effects and habitat degradation reduces seedling densities which appear to be associated with increased canopy disturbance. These findings are consistent with the observed low frequency of recruitment of *D. malabaricum* especially from the pole stage to the adult stage (>5 cm DBH) as indicated by the data on population structure. Below we discuss the evidence for the genetic and ecological factors influencing the recruitment and survival of *D. malabaricum*. We make recommendations for management and restoration which are not only relevant to this species but could be applied to other ecologically similar tropical hardwood species surviving in agro-forest landscapes.

<table>
<thead>
<tr>
<th>Genetic factors influencing seedling performance in <em>D. malabaricum</em></th>
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| Our results demonstrate that seedlings raised from seeds collected in low density forest patches have elevated inbreeding and lower growth performance than those from high density patches. We interpret this as a sign of inbreeding depression leading to reduced seedling performance (average growth rates) under nursery conditions in *D. malabaricum* progeny. Our previous study demonstrated that more frequent mating among related individuals occurs when adult *D. malabaricum* densities are reduced (Ismail *et al.* 2012). This study confirms that the increase in inbreeding associated with fragmentation reduces fitness of seedlings, and thus may influence recruitment. Our estimates of bi-parental inbreeding provide evidence for the potential importance of inbreeding depression driving this reduced performance (Angeloni *et al.* 2011). Alternative mechanisms might be low pollen diversity (Breed *et al.* 2012b) or maternal origin (Cascante *et al.* 2002) but we have no evidence to support these explanations.

<table>
<thead>
<tr>
<th>Evidence for recruitment failure in <em>D. malabaricum</em></th>
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</table>
| We observe relatively high seedling and sapling frequencies across our survey plots. However, pole stage trees were extremely rare. This suggests that despite significant seed production and germination only a small fraction of saplings are surviving to the pole stage. We observed an almost complete absence of the smallest (>5cm < 20 cm DBH) trees across the entire 216 km² study area (Figure 2). Based upon preliminary dendrological investigations in this species (see Appendix S3 in Supporting Information for details), we estimate that trees greater > 20 cm DBH are approximately 30-40 years old, which would just predate the last expansion of coffee plantations in the region (Garcia *et al.* 2010). For trees less than 60 cm DBH we interpret the constant decline of abundance from one size class to the next smaller size class as declining recruitment
success over time dating back to the intensification of commercial logging and plantation crops in the Western Ghats some 80-100 years ago (Chandran 1997). Taking all these factors into account we infer that recruitment of *D. malabaricum* is extremely low and appears to be declining in the forest patches we have studied. The observed variation of seedling and sapling densities across patches does not correspond with the density of reproductive adult trees (no significant fit to the MLE) suggesting that seed production *per se* is not driving the limited recruitment. However, a lack of transition of individuals from the sapling to the pole stage might be a major limitation for recruitment. Long lived tree species might recruit at episodic intervals spanning several years, however, the size class distribution indicates that for at least 30 years (a period much longer than normal episodic recruitment) conditions have not favoured pole stage recruitment.

<table>
<thead>
<tr>
<th>Indicators of habitat degradation and implications for <em>D. malabaricum</em> seedling densities</th>
</tr>
</thead>
</table>
| Our evaluation of habitat suitability suggests that degradation of forest patches as indicated by increased openness of dense old growth forest is leading to sites less favorable for *D. malabaricum* recruitment. We base this view upon three main lines of evidence: a) density of *D. malabaricum* seedlings is negatively associated with the density of *C. viscosum* seedlings; b) density of *D. malabaricum* seedlings is negatively associated with the percentage of open canopy and; c) density of *D. malabaricum* seedlings is positively associated with the density of arboreal termite nests. *C. viscosum* is an early successional light demanding species establishing after gap formation (Chandrashekara and Ramakrishnan 1994) and becomes increasingly dominant in forest where the canopy is opened. It is noteworthy that *C. viscosum* seedling density and canopy openness do not correlate significantly. The most likely explanation for this is that sub-canopy closure (which is above the height at which we measure openness) has already occurred when early successional species such as *C. viscosum* become well established. That *D. malabaricum* seedling density declines in more open canopy forest patches, could be due to increasing abundance of more competitive light demanding species, or because the microclimate is less suitable.

It is interesting to note that *D. malabaricum* seedling densities are greater where arboreal termite nests are more abundant, which may signify more favorable habitat. Termite communities were shown to be effective biological indicators of forest degradation in other regions because they are sensitive to forest disturbance, especially to canopy loss (Eggleton et al. 1996; Jones et al. 2003; Alves et al. 2011). Termites are also important agents of soil formation and nutrient cycling in tropical forests (Ghazoul and Sheil 2010), which may additionally influence local edaphic conditions. Drawing any direct causal links between the evaluated variables...
and *D. malabaricum* seedling densities is beyond the scope of this data. However, the associations provide a useful signal that habitat changes affect recruitment of this species.

One additional factor which we recorded but was ultimately excluded by the final model selection was density of coffee seedlings. There was no overall statistically significant relation between this and *D. malabaricum* seedlings across all our plots. However, it is noteworthy that in plots where we observe high densities of coffee seedlings (>200 per 100 m²) we detect consistently low densities of *D. malabaricum* seedlings. These plots had on average more than six times lower *D. malabaricum* seedling densities than the other plots. The potential competitive interactions between *D. malabaricum* and coffee seedlings are worthy of empirical validation.

**Applied management and policy recommendations**

Agricultural landscape mosaics which retain forest features offer great promise as habitats to support a wide array of tropical biodiversity (Perfecto and Vandermeer 2008). The long-term benefits of these landscapes largely depend on the persistence of forest patches, which provide critical habitats and resources for many forest dependent species. Our results demonstrate that conversion of forest patches to alternative land-use and subsequent fragmentation is likely to have direct but invisible genetic consequences on the constituent forest tree species. The current but slow degradation of remnant forest represents insidious changes, which additionally undermine recruitment of old-growth tree species. Conservation management needs to address both of these factors if these agro-forest landscapes are to sustain their many ecological functions.

Despite the relatively large number of private forest within our 214 km² study area, we found no adult *D. malabaricum* trees in these forest patches. The vast majority of trees were found within so-called ‘Sacred grove forests’ which are small remnants of native forest conserved by the local community for worshipping (Chandrakanth et al. 2004). Very few trees (5%) were found in coffee plantations. Presumably *D. malabaricum* was already logged from private forests some time ago because of their high market value. Collection of forest resources from sacred groves is traditionally restricted to fruits and leaves for ritualistic uses (Chandrakanth et al. 2004). However, the general erosion of cultural affinity with the sacred grove forests has led to further forest degradation (Garcia C.A. and Pascal J.P. 2006; Ormsby and Bhagwat 2010). Enforcing traditional usage rights within the sacred groves would help reducing degradation by extensive fire wood collection and especially preventing illegal logging causing canopy gaps. In addition, artificial seeding of these forest patches and even private forest with outbred *D. malabaricum* seed might help to improve seedling vigour and reduce fragmentation in the
longer term. Outbred seed can be obtained from high density populations and single isolated trees which have been shown to capture very heterogeneous pollen from different trees over large distances (Ismail et al. 2012).

Where old growth canopy trees species have been logged within sacred groves (village temple committees are allowed, with Forest Department consent, to extract timber for temple construction and repair (Chandrakanth et al. 2004)) special efforts will be required to ensure that these trees are replaced. Indeed, recovery of *D. malabaricum* in private forests is extremely unlikely based upon our study. Planting nursery raised seedlings to augment the density of *D. malabaricum* and accelerate succession, together with protection and regular maintenance of planted trees may be necessary to ensure they survive and become reproductive adults both in sacred groves and in private forest. This would enhance genetic connectivity thus mitigating the negative genetic consequence of low local adult tree densities (Ismail et al. 2012).

**| Conclusion**

Conversion of tropical forests to alternative land use, subsequent fragmentation and habitat degradation are pervasive across the tropics. The decline of large canopy trees is a global phenomenon predicted to seriously undermine ecosystem integrity (Lindenmayer et al. 2012). Our findings not only inform the management of one important timber species but are relevant to other rare canopy trees. For example, *Artocarpus hirsutus* and *Canarium strictum* are two threatened species within our study area known to experience low seed germination and high seedling mortality in small forest patches (Tambat et al. 2005). A failure to conserve this important biodiversity within forest patches is likely to have implications for ecosystem services. Large old trees such as those above are not only important carbon stores but provide critical nesting sites for many threatened bird species and for the giant honey bee (*Apis dorsata*) which deliver economically substantial pollinator services to the surrounding coffee plantations in the region (Krishnan et al. 2012). If complex agro-forest landscapes are to sustain both biodiversity and other ecosystem services in the future, the ecological and genetic consequences of degradation and fragmentation must be considered to ensure persistence of large old growth tree species.

**| Acknowledgements**

We thank the hard-working field assistants Navin H. Kumar, Monappa C.S., Chengappa S.K. and particularly Range Gowda, Harsha C. and the late Umesh who took care for the nursery experiment in the absence of S.A.I. The painstaking single DNA extractions conducted by Sandeep Sen and Shruthi Jayappa are esteemed a lot. Fragment analysis was conducted at the Genetic Diversity Centre (GDC) of ETH Zürich. This research was funded by ETH Zürich under the grant number ETH-22 08-2.
REFERENCES


## SUPPORTING INFORMATION

**Table S1: Details of Dysoxylum malabaricum seed sampling for the nursery experiment**

<table>
<thead>
<tr>
<th>Site name</th>
<th>Total seed planted per site</th>
<th>Total survived 21 months per site</th>
<th>ID of nearest adult tree</th>
<th>Total seed planted per adult tree</th>
<th>Total survived 21 months per adult tree</th>
</tr>
</thead>
<tbody>
<tr>
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Appendix S1: Methods of genotyping and genetic analysis

We collected after 21 months of growth leaf tissue which was desiccated with silica gel prior to DNA extraction. DNA was extracted using a CTAB (cetyltrimethyl ammonium bromide) extraction (Sambrook et al. 1989). We genotyped 297 seedlings raised in the nursery at eleven polymorphic 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 et al. 2010). The polymerase chain reaction (PCR) conditions were as described in Ismail et al. 2012. Amplified fragments were analyzed on an ABI3730 (Applied Biosystems) capillary sequencer and scored relative to a LiZ500 HD size standard with GeneMapper 3.5 (Applied Biosystems). Over the 297 seedling samples we detected between 4 and 17 alleles per microsatellite locus and a total of 112 alleles with a proportion of 99.4% of loci genotyped. The genetic data of the 235 adult trees from 37 sites and the 488 seedling samples collected from 19 forest patches are taken from Ismail et al. (2012), where also details of the sampling and genotyping are described. To compare basic values of genetic diversity among the different cohorts (nursery samples, adults and seedling samples) we calculated observed and expected heterozygosity using GENEALEX 6.41 (Peakall and Smouse 2006). Multilocus allelic richness (and inbreeding coefficients (Fis) was estimated with FSTAT 2.9.3.2 (Goudet 1995).

To detect the two most likely parents we applied parentage analysis with the delta maximum-likelihood approach implemented in CERVUS (Marshall et al. 1998).

We ran simulations of parentage based on the allele frequencies of the adult trees with the following parameters: 10 000 simulated offspring with all 235 adult trees as candidate parents, estimated to represent 98% of the parents within the area (allowing for selfing). The minimum of matching loci was set at six; the error rate of mistyped loci was set 0.01 and the proportion of loci genotyped was 0.98. The significance threshold for the assignment of the two most likely parents was set at 90%.

Appendix S2: Details of the statistical analysis

Evaluation of inbreeding under nursery conditions

To investigate whether genetic effects (measured as kinship of parent pairs and individual inbreeding coefficient) affect seedling performance we analyzed whether levels of inbreeding and fragmentation affect seedling sizes over a period of 21 months of growth. Because model assumptions like variance homogeneity are not met, linear regressions of levels of inbreeding against performance were not applied. As an alternative we explored the potential for contras-
ting local adult tree density to influence inbreeding and performance in our nursery seedlings. The effect of local tree density in a forest patch has already been demonstrated to influence patterns of inbreeding in *D. malabaricum* (Ismail *et al.* 2012).

We classified the nursery seedlings into those collected from forest patches of low density (LD) and those from high density patches (HD). Nursery seedlings were classified as LD when there are less than 6 adult trees within 500 meters for any tree within the forest patch of seed collection. Consequently provenances with more than 6 adult trees within 500 meters for any tree within the forest patch of sample collection were defined as HD, these correspond with our categories for fragmentation. We tested for significant differences of average seedling size, average individual inbreeding coefficient and average kinship coefficient between LD seedlings and HD seedlings using non parametric Wilcoxon rank sum test.

| Evaluating ecological degradation and recruitment of *D. malabaricum* |

All statistical analysis were performed with R 2.13.1 (R Development Core Team 2011). To investigate reproductive success of *D. malabaricum* in the wild, MLRs were performed with seedling densities as the response variable and as explanatory variables, the variables on habitat degradation. To determine the ecological degradation of each forest patch and to account for the nestedness of the study design, the measurements of the five plots per forest patch were averaged before analysis. This resulted in 17 data points. Averaging the data has the advantage that we avoid the statistical limitation of zero inflation (36 plots with zero seedlings and 61 plots with zero saplings) in the response variable and that we can include potentially important variables recorded at the patch level which are number of adult *D. malabaricum* trees, the proportion of the forest border which is adjacent to coffee plantations and the area of the forest patch.

The initial MLR model included as predictor variables; percentage of closed canopy, coffee seedlings, *C. viscosum* seedlings, termite nests, the proportion of the forest patch border adjacent to coffee plantations number of adult *D. malabaricum* trees and the area of the patch against the seedling abundance as dependent variable. To meet model assumptions seedling densities were square root transformed. All explanatory variables were tested to be independent to avoid autocorrelation. On this initial model we applied forward as well as backward model selection with the step function implemented in R to identify and exclude irrelevant variables and to improve the model.
Appendix S3: Dendrological estimates

Although age estimates of tropical trees are difficult we approximate the larger size class trees (DBH 60-100 cm) of adult *D. malabaricum* trees in our study area to be between 100 to 160 years old. This is based upon tree ring counts of two felled *D. malabaricum* trees of 88cm and 104cm DBH which had 244 rings and 332 rings respectively. Because tree rings in tropical trees are rarely strictly annual (Worbes 2002) and because yearly rings result in unreasonable old ages we assume a maximum of two rings per year (one during the monsoon season and one during the drought season). Given the strict seasonality in the region this assumption seems plausible because both long periods of inundation and drought produce tree rings in tropical trees (Worbes 2002). We estimate a diameter growth rate of approximately 3-4 mm per year which is in the range of the highest published growth rates for the same region (Rai 1981). This approximation thus represents a minimum age estimate.

References (supporting information)


CHAPTER 5

Genetic responses of Vateria indica to spatial isolation: Implications for seed sourcing and forest restoration

with J. Ghazoul | G. Ravikanth | R. Uma Shaanker | C.G. Kushalappa | C.J. Kettle |

ABSTRACT

Tropical agro-forest landscapes might provide repositories of genetically diverse sources of timber tree germplasm important for restoration of degraded forests. The Dipterocarpaceae are a main timber tree family in Asia, as dominant canopy species. Dipterocarps might be especially vulnerable to habitat fragmentation due to limited seed dispersal and significant fine-scale spatial genetic structure. This study provides a baseline on the importance of fragmented agro-forest landscapes for conserving the genetic resources of an important dipterocarp species in India.

We compare levels of genetic diversity, inbreeding and fine-scale spatial genetic structure (FSGS) in an isolated and a continuous population of the threatened timber tree species Vateria indica within an agro-forest landscape in the Western Ghats, South India using twelve nuclear microsatellite markers. We apply parentage analysis of genotyped seed, nursery raised seedlings and established seedlings to estimate pollen and seed dispersal. Using a nursery trial we evaluate effects of inbreeding on growth performance of seedlings.

Our results show that levels of FSGS, pollen dispersal and seed dispersal are comparable between a small isolated and large continuous population of V. indica, although initial sampling effects of fragmentation explain lower diversity in the smaller population. Occasional realized long distance pollen flow into the isolated population helps maintaining genetic diversity. The comparison of inbreeding between seed and seedlings and the nursery experiment suggest selection in favour of outbred progeny under field conditions due to reduced vigour of selfed seedlings. V. indica has a more intense FSGS compared to three dipterocarp species from Borneo but lower FSGS than the highly fragmented and rare Vateriopsis seychellarum. We conclude by providing recommendations for sourcing germplasm in V. indica for restoration and discuss the wider implications of our findings in the context of conservation and restoration of dipterocarp forest in agro-forest landscapes.
INTRODUCTION

Tropical agricultural landscape mosaics which include significant numbers of forest trees offer great potential as centres of biodiversity outside of continuous protected forest (Perfecto and Vandermeer 2008; Anand et al. 2010). These novel landscapes are generally highly fragmented with spatially disrupted tree populations and individual trees which are isolated from conspecifics. Fragmentation might reduce the number of reproductive trees, alter pollen and seed dispersal patterns and affect the reproductive output of plant species (Aizen and Feinsinger 1994; Ghazoul 2005). In addition to these direct consequences for reproduction of individual species, there are genetic consequences including loss of genetic variability, increased genetic drift and inbreeding (Lowe et al. 2005; Aguilar et al. 2008) which are known to be important factors in accelerating species and population extinction risk (Spielman et al. 2004; Frankham 2005; O’Grady et al. 2006).

The erosion of tree diversity in tropical landscapes is likely to have considerable consequences for biodiversity at higher trophic levels through cascade effects (Manning et al. 2006) and ultimately leads to a reduction in the biodiversity value of such landscapes. Predictions on the consequences of forest fragmentation and of the quality of the intervening matrix for biodiversity in human dominated landscapes (Koh et al. 2010) generally do not consider genetic biodiversity despite its fundamental importance for species persistence (Laikre et al. 2010) as well as for ecosystem functioning by directly supporting biodiversity (Bailey 2011). Understanding how effective such fragmented landscape mosaics are at conserving genetic diversity of the forest tree species they harbor is pertinent for overall biodiversity conservation. In particular, diverse agro-forest landscapes might provide important repositories of genetic diversity for many valuable timber tree species which are not only important for future sustainable timber production but essential for restoration of highly degraded forest landscapes.

There is a substantial body of research demonstrating that the genetic consequences of fragmentation for tropical tree species is highly idiosyncratic and can show both positive (Dick 2001; White et al. 2002; Jha and Dick 2010; Ottewell et al. 2010), and negative responses (Stacy et al. 1996; Garcia et al. 2005; Breed et al. 2012; Ismail et al. 2012b). One globally important family of tropical trees is the Dipterocarpaceae, which dominate the forest canopy of Southeast Asia, and account for a quarter of the global trade in tropical hardwoods (ITTO 2008).

The ultimate objective of this study is to advance our understanding of how genetic resources of an important dipterocarp species in India can be conserved within a fragmented agro-forest
landscape. We investigate the effect of spatial isolation on genetic structure and gene flow within a complex landscape mosaic that includes a large more continuous population and a smaller isolated population of *Vateria indica*, a critically endangered timber species endemic to the Western Ghats, South India (Ashton 1998). The study area is located in Kodagu district in a highly fragmented agro-forest landscape dominated by coffee plantations and paddy (Figure 1).

We evaluate genetic diversity, fine-scale spatial genetic structure (FSGS) inbreeding and patterns of seed and pollen dispersal in this landscape using 12 nuclear microsatellite loci and a sample of 240 adult trees and 694 progeny to elucidate the consequences of fragmentation for the underlying genetic resources. Using adult trees and known differences in diameter at breast height (DBH) as a surrogate for age, we conducted a parentage analysis of the youngest adult trees, using all larger adults as candidate parents. This enables us to investigate signs of temporal changes in intensity of fragmentation. In addition we compare genetic parameters from nursery-raised seedlings with wildlings (forest seedlings) populations to allow us to investigate whether inbreeding has negative consequences on growth performance.

Using the above framework we address the following research questions: 1) Does a small isolated population retain comparable levels of genetic diversity to a larger more continuous population? 2) Do patterns of gene flow and consequently genetic structure differ across life stages and between the continuous and isolated population. 3) What are the recommendations for conservation of genetic diversity in fragmented *V. indica* populations based upon our findings? This research has broad relevance for the persistence of many tree species restricted to forest fragments in human dominated landscapes. More specifically, it will add to the body of research on the many overexploited and highly fragmented timber tree species within the Dipterocarpaceae family. The dipterocarps provide an interesting family in the context of forest fragmentation as they exhibit limited seed dispersal by gyration or gravity and fine-scale spatial genetic structure (FSGS) is commonly observed in the family (Kettle *et al.* 2011; Harata *et al.* 2012), and very little is known about the implications of fragmentation for reproduction in this family (Kettle *et al.* 2012). Intense FSGS can increase species susceptibility to fragmentation because it can promote genetic isolation of related individuals and consequently consanguineous matting. Therefore we compare the intensity of FSGS observed in this study with FSGS of four other dipterocarps based on published data by Kettle *et al.* (2011) and Finger *et al.* (2012).

In many predominantly outcrossing plant species it was shown that spatial isolation can change matting patterns towards increased selfing. Selfing is an important means of reproductive assurance in the absence of pollinators or mating partners in some species (Eckert *et al.* 2010;
Levin 2010). Increased selfing rates in response to fragmentation in dipterocarp species were shown to increase inbreeding in mature seed (Obayashi et al. 2002; Naito et al. 2008) as well as in wildlings (Fukue et al. 2007; Finger et al. 2012). In addition, because seed of dipterocarp species are recalcitrant and unlikely to reach habitat patches where they have been extirpated, management of germplasm for enrichment planting or restoration through artificial seeding or planting is of great relevance. Our results provide important information for seed sourcing and management of genetic resources in human dominated landscapes which can be directly applied in conservation and forest restoration.
METHODS

Study area and study species
The study sites are located in the Western Ghats biodiversity hotspot within Kodagu district, South India (Figure 1). This district is a major coffee growing region contributing to one third of the Indian coffee production (Coffee Board of India 2008 in Garcia et al. 2010). Within this district we focus on two populations of *Vateria indica* surrounded by an agricultural matrix consisting of shade coffee plantations and paddy. One population occurs within two forest patches separated by 320 metres where we located 68 adult trees and 14 adult trees plus 3 singleton trees in the surrounding coffee plantations. These two patches are dominated by the focal species with 61 *V. indica* trees per ha. The second population is located 2.3 km west of the small isolated population. This population of *V. indica* is restricted to a river bank bordered by shade coffee plantations and represents the nearest population to the isolated population. In this population we sampled 155 adult trees along a 2.4 km stretch of river running from north to south. Because of difficult access and because of increasing abundance of *V. indica* along the river we could not sample all the individuals. We consider these trees as part of a continuous population as the population extends along the river further towards the south but not to the north. We know of more *V. indica* populations within 1.5 km to the west of the continuous population.

Figure 1: Overview map of India with Karnataka (dark grey) and Kodagu District (white) and in the box a zoom of the study area. Dots show adult Vateria indica trees with the isolated population on the right and the continuous population on the left located along the river indicated with the line.
V. indica is endemic to the Western Ghats and an integral species of the most western lowland dipterocarp forests dominated by V. indica and Dipterocarpus indicus (Pascal and Pelissier 1996). Vateria indica is listed as critically endangered on the 2012 IUCN Red List based on its overexploitation for its timber and its habitat loss of more than 80% (Ashton 1998). The species was shown to be suitable for restoration of degraded wet evergreen rain forests due to its good survival and a rather fast growth of 1 cm to 1.5 cm girth and 30 cm to 31 cm height per year investigated over periods of 12 to 44 years (Rai 1990). When water availability is good V. indica is canopy emergent (30 – 50 m) while it is canopy forming at 10-30 metres in less favourable conditions (Pascal and Pelissier 1996). The species is almost strictly biannually flowering with white fragrant flowers with c. 18 mm calyx width. The genus Vateria is known to be principally bee pollinated (Ashton 1988). Indeed we frequently observed Apis dorsata and less frequently Apis cerana to forage on the flowers. The wingless and normally one seeded fruit is ripe during peak monsoon in July and August (Murali 1997). The fruit weighs 12.5 gr (Murali 1997) and is expected to drop near the mother tree as there is no dispersal agent known.

| Sample collection |
For all our sample collections we defined adult trees by their diameter at breast height (DBH) of more than 10 cm. This threshold was chosen because we observed that at this stage branches become orthotropic. This orthotropic branching indicates the transition to a mature flowering tree (Durand 1997). Wildlings were defined by their maximum size of 50 cm. In the small isolated population we mapped with a Garmin 60CSx handheld GPS all the adult trees on an accuracy of five metres. Of the 85 located trees we collected leaf or cambium tissue. We collected progeny samples from this population in the 2009 fruiting season. We randomly selected 30 adult trees and collected 20 fresh fruits from the ground within 10 metres of each putative mother. Half of the fruit were used for immediate genetic analysis and half of the fruit were planted in a nursery for subsequent comparison of genetic diversity after germination. The former resulted in 300 fruit samples which were dissected within 24 hours to obtain pure embryo tissue. The latter 300 fruit samples plus 10 fruits collected under an additional tree were planted within two days into 2 litre polyethylene bags filled with a mixture of one part local red soil, two parts cow dung and two parts river sand and kept under a 50% shade cloth. Of the initially planted 310 fruit 228 germinated (of which 40 were lost due to ants which nested in the seed as soon as the seed coat split) and 202 survived for 21 months. From the surviving seedlings we collected leaf tissue. To prevent local effects, such as daylight orientation and neighbour competition, the seedlings were rotated monthly within the shade house. To reduce mortality due to a caterpillar infestation we applied once insecticide uni-
formly across the plants. Seedling growth was measured monthly from soil surface to top of the apical meristem with an accuracy of 0.5 cm.

In May 2012 we mapped and sampled leaf tissue of 120 wildlings, five within 10 metres of 24 randomly selected trees in the isolated population. To exclude wildlings from the 2011 fruiting event we excluded individuals that were still attached to the remains of their seed coat. This approach maximised the probability of sampling wildlings only from the 2009 fruiting event.

In the larger continuous population we sampled and mapped 155 adult trees along a river bed running from north to south in April 2010. Within the first 1.6 km we sampled 79 individuals which was an exhaustive sample of the *V. indica* adult trees along this stretch of river. From that point up to 2.4 km we sampled every 200 metres within a radius of 50 meters up to 20 individuals, because the abundance of trees was too great to sample all. Additionally, we followed at 2 km a small stream 180 metres into an adjacent coffee plantation along which we sampled another 18 individuals. Within the most southern 0.8 km we sampled 76 adult trees which represent certainly more than 50% of the trees that were present. All trees were sampled for cambium or leaf tissue. For the wildling sampling we collected leaf tissue from a maximum of 20 individuals within a 10 metre radius of each of six selected putative mother trees. The wildling sampling sites were selected to be approximately evenly spaced along the river stretch. The distances from one selected tree to the next from north to south were two times 500 m and 3 times 250 m.

| Genotyping |
All tissue samples except the nursery samples were lyophilised with silica gel for storage prior to DNA extraction and subsequent genotyping. The nursery samples were frozen at -20°C before processing. Genomic DNA was extracted from the tissue samples with a CTAB-method (Sambrook et al. 1989) in the conservation genetic laboratories of the Ashoka Trust for Ecology and Environment (ATREE). The genotyping was based on 12 nuclear microsatellite markers (Vi93, Vi15, Vi16, Vi55, Vi45, Vi13, Vi60, Vi91, Vi18, Vi78, Vat14 and Vat10) shown to be suitable for population genetic studies of *V. indica* (Ismail et al. 2012a). The microsatellite loci were amplified following the PCR conditions described in Ismail et al. (2012a). The amplified fragments were analysed in an ABI3730 capillary sequencer and scored relative to a LIZ 500 HD size standard in GeneMapper 3.5 (Applied Biosystems). All DNA samples from adult trees were successfully genotyped at eight or more loci. Of the DNA samples from wildlings of the isolated stand, the wildlings from the continuous stand and of the nursery samples between 98% and 99% amplified at eight or more loci. Because DNA extraction from the seed did not yield the
same DNA quality as the leaf samples, only 259 samples of the 310 DNA samples amplified at eight or more loci (See Table 1 for the successfully at eight or more loci genotyped samples across the different sample locations and life stages).

**Genetic analysis**

**Levels of genetic diversity, inbreeding and differentiation**

Deviations from Hardy-Weinberg Equilibrium (HWE) and frequency of null alleles and at each locus, were calculated in CERVUS 3.0.3 (Marshall et al. 1998) for the adult samples from the continuous population and the isolated population. For the adult populations, the wildling samples, the seed and the nursery samples we calculated the mean number of alleles (Na), the observed heterozygosity (Ho) and the expected heterozygosity (He) with GeneAlEx 6.5b3 (Peakall and Smouse 2006). Additionally we estimated the inbreeding coefficient (Fis), the allelic richness (Rs) and the pairwise levels of differentiation (Fst) with FSTAT 2.9.3.2 (Goudet 1995) using 1000 permutations.

**Estimating gene flow patterns**

To directly estimate realized gene flow we applied the delta maximum-likelihood exclusion analysis to assign the two most likely parents to the juvenile samples with CERVUS 3.0 (Marshall et al. 1998; Kalinowski et al. 2007). Additionally, we also investigated if the smaller adult trees within the isolated stand derive from larger trees within this stand. For this analysis we treated the 73 trees with a DBH of more than 50 cm as potential parents and the 12 trees with a smaller DBH as potential progeny. The combined non-exclusion probability for a parent pair of our marker set is 5.3 x 10^{-7}. Simulations of parentage were based on the allele frequencies from all the 240 adult trees from both populations which served as candidate parents. We simulated 10000 offspring with the following parameters: the minimum number of typed loci was set at six, the error rate of mistyped loci was set at 1% with 99.3% of the loci genotyped and allowing for selfing. The parameter settings of the parentage simulations differ between the wildlings of the continuous population and the offspring samples from the isolated population only by the number of sampled candidate parents which was set as 80% and 95% respectively. The confidence level for the assignment of the two most likely parents was set at 85%. Special attention was given to assignments where both putative parents beyond were 100 meters from the wildling location. Because these assignments imply unlikely long seed dispersal distances, we considered those events only if they were assigned to two parents at the 90% significance threshold.
Pollen dispersal was calculated for each offspring sample as the distance between the two most likely parents. Because the seed are primarily dispersed by gravity we assume that pollen dispersal always exceeds seed dispersal (except in the case of selfing). Thus the seed dispersal distance was calculated as the distance between the wildling and the nearer parental tree following Finger et al. (2010).

To investigate if historic levels of gene flow differ between the two adult populations and to investigate temporal changes in gene flow we additionally estimated overall levels of historic gene dispersal distances (σ) of the two adult populations based on FSGS. These estimates were computed with SPAGeDi 1.3a (Hardy and Vekemans 2002) based on 10000 permutations of locations and genes. Estimates of σ are sensitive to the effective density of the population. Effective density can be estimated by multiplying the census density by the ratio of effective population size to the census population size (Hardy and Vekemans 2002). Demographic studies demonstrated that effective population sizes in plant populations range approximately from half to a tenth of the census population (Frankham 1995). Based on natural densities (Dn) of adult V. indica trees we thus estimated a range of σ based on Dn/2 and Dn/10 as estimates of effective population densities. Because the census densities of the studied populations are unlikely to represent their historic densities we estimated Dn as 104 V. indica trees (DBH>10 cm) per ha based on a former study in a 28 ha permanent plot located in pristine unlogged forest within Kodagu district (Pascal and Pelissier 1996).

Patterns of FSGS
To evaluate the levels of FSGS we investigated the correlation of pairwise relatedness (r) and the kinship coefficient (F) (Loiselle et al. 1995) over the pairwise spatial distance of the samples with GenAlEx (Peakall and Smouse 2006) and SPAGeDi 1.3a (Hardy and Vekemans 2002). The levels of FSGS were computed separately for the adults of both populations and the wildlings of the isolated population. We defined six distance classes for the pairwise comparisons of kinship up to 640 metres (end point of each distance class is 10, 20, 40, 80, 160 and 640 meters). The distance classes were chosen to ensure in all three cohorts sufficient pairwise distances. The minimal number of pairwise distances was 58 pairs in the shortest distance class of the adults from the isolated population. The wildlings from the continuous population had to be excluded because of insufficient numbers of pairwise distances between 20 and 160 meters. For comparing the intensity of FSGS between the two adult cohorts and the wildlings we calculate the Sp-statistic (Vekemans and Hardy 2004), defined as 

$$S_p = \frac{-\hat{b}_r / (1 - \hat{F}_r)}{1}$$

where \(\hat{b}_r\) is the mean regression slope of the kinship coefficient over the natural logarithm (ln) of the distance and \(\hat{F}_r\) is the mean kinship of the sample pairs within the first distance class (0-10 m). To test...
if relatedness at the different distance classes differs significantly we computed the squared paired-sample t-test statistic ($t^2$) described as single class heterogeneity test in (Smouse et al. 2008) and implemented in the program GenAlEx 6.5b3 (Peakall and Smouse 2006). Significance of p values of the heterogeneity test was only considered when p<0.01 as recommended in (Banks and Peakall 2012). We further compare the intensity ($\hat{F}$ and SP) of the observed FSGS with four other dipterocarp species. For these comparisons we reanalysed the present data and the data published in (Kettle et al. 2011) and Finger (Finger et al. 2012) using 20, 40, 80, 160, and 1280 meters as the end points of distance classes.

**Effects of inbreeding on seedling performance**

Based on the parentage analysis of the nursery raised seedlings (described above) we calculated the kinship coefficient (Loiselle *et al.* 1995) of the two most likely parents as a measure of inbreeding in SPAGeDI 1.3a (Hardy and Vekemans 2002). To investigate the effect of inbreeding on growth performance we split up the nursery raised seedlings into outbred and selfed progeny. The outbred seedlings were further split into two groups at the median of their kinship coefficients to investigate the effect of mating between more related individuals. Differences in size variation after 21 months of growth among the 3 groups of seedlings were tested with an ANOVA. To investigate for significant differences among the mean sizes the three groups of samples we applied a post hoc Tukey HSD test implemented in R 1.13.1 (R Development Core Team 2011).
RESULTS

| Genetic diversity, inbreeding and differentiation |

At the 12 loci across all samples we detected between five and 15 alleles with a total of 117 alleles. When estimating the deviations from HWE and null allele frequencies based on the adult samples from the continuous population only we detected no evidence for any deviations from HWE and very low frequencies of null alleles (with 0.08 in locus Vi45 having the highest estimate). The same analysis applied to the small isolated population revealed a homozygous excess at two loci, locus Vat14 (of 0.49 null alleles) and locus Vi18 (null allele frequencies of 0.18). Deviations of HWE could not be calculated for locus Vat14 due to the low frequencies of some alleles (which was set at five in CERVUS). Additionally we observed significant deviation from HWE at locus Vi18. Because we can rule out differences in the processing of the samples we interpret these findings as genuine deviations from HWE. Indeed of the four alleles detected at locus Vat14 one allele contributes 90%. At Vi18 we observe a similar pattern where one allele contributes to 50% of the 5 detected alleles.

Genetic diversity measured as number of effective alleles, expected heterozygosity and allelic richness within the large continuous population and within the small isolated population remains at similar levels when comparing adults and wildling. In this population the seed samples and the nursery raised seedlings show a slightly reduced allelic richness compared to adults. When comparing genetic diversity between the two populations, the isolated population has significantly lower genetic diversity across all life stages estimated from double standard errors (Table 1).

When comparing the inbreeding coefficients ($F_{is}$) of adults and wildlings within populations they do not differ much. The adults and wildlings from the isolated population and the wildlings from the continuous population do not differ significantly from zero based on the 95% confidence level. The $F_{is}$ value of the adult cohort from the river population differs marginally from zero. Interestingly the $F_{is}$ values of the nursery and the seed samples from the isolated population are significantly higher than zero and higher than the $F_{is}$ values of the wildlings and adults within this population (Table 1).
Table 1: Mean values of genetic diversity parameters and inbreeding coefficients in *Vateria indica* across life stages from a continuous and an isolated population.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>( \bar{\theta} ) no. of alleles</th>
<th>( H_o )</th>
<th>( H_e )</th>
<th>Allelic richness * small s (Fis)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SIP Adults</strong></td>
<td>85</td>
<td>5.25</td>
<td>0.56</td>
<td>0.576</td>
<td>5.21 (0.024)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.52</td>
<td>±0.068</td>
<td>±0.057</td>
<td>±0.52 (-0.05; 0.116)</td>
</tr>
<tr>
<td><strong>LCP Adults</strong></td>
<td>155</td>
<td>8.5</td>
<td>0.656</td>
<td>0.69</td>
<td>7.9 (0.041)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.60</td>
<td>±0.037</td>
<td>±0.033</td>
<td>±0.60 (0.015; 0.068)</td>
</tr>
<tr>
<td><strong>SIP Wildlings</strong></td>
<td>117</td>
<td>5.92</td>
<td>0.583</td>
<td>0.603</td>
<td>5.72 (0.024)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.47</td>
<td>±0.051</td>
<td>±0.051</td>
<td>±0.45 (-0.009; 0.056)</td>
</tr>
<tr>
<td><strong>LCP Wildlings</strong></td>
<td>119</td>
<td>7.83</td>
<td>0.643</td>
<td>0.708</td>
<td>7.42 (0.072)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.82</td>
<td>±0.047</td>
<td>±0.027</td>
<td>±0.74 (-0.007; 0.153)</td>
</tr>
<tr>
<td><strong>SIP Seed</strong></td>
<td>259</td>
<td>0.08</td>
<td>0.492</td>
<td>0.608</td>
<td>4.77 (0.155)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.45</td>
<td>±0.043</td>
<td>±0.045</td>
<td>±0.40 (0.096; 0.211)</td>
</tr>
<tr>
<td><strong>SIP Nursery seedlings</strong></td>
<td>199</td>
<td>5.58</td>
<td>0.513</td>
<td>0.611</td>
<td>5.07 (0.11)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±0.50</td>
<td>±0.042</td>
<td>±0.042</td>
<td>±0.42 (0.056; 0.161)</td>
</tr>
</tbody>
</table>

* Standard error, figures in brackets are 95% confidence intervals obtained from jackknifing over loci.
* *Based on 77 diploid

The pairwise comparisons of \( F_{st} \) among the populations and cohorts show that only the adults and their wildlings from the isolated population as well as the seed and nursery samples do not differ significantly from each other. All the other pairwise \( F_{st} \) values differ significantly at the 5% level which is adjusted for multiple comparisons 0.3% (Table 2).

Table 2: Pairwise *F*<sub>st</sub> values across populations and cohorts of *Vateria indica*. *indicates p values below 0.003 which is the adjusted nominal level for multiple comparisons at the 5% level, SIP: small isolated population, LCP: large continuous population.

<table>
<thead>
<tr>
<th></th>
<th>SIP adults</th>
<th>LCP adults</th>
<th>SIP wildlings</th>
<th>LCP wildlings</th>
<th>SIP seed</th>
<th>SIP nursery</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIP adults</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCP adults</td>
<td>0.071*</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIP wildlings</td>
<td>0.0012</td>
<td>0.062*</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCP wildlings</td>
<td>0.1081*</td>
<td>0.024*</td>
<td>0.0979*</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIP seed</td>
<td>0.0097*</td>
<td>0.0639*</td>
<td>0.0129*</td>
<td>0.1013*</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>SIP Nursery</td>
<td>0.0142*</td>
<td>0.0691*</td>
<td>0.0162*</td>
<td>0.1026*</td>
<td>0.0019</td>
<td>0</td>
</tr>
</tbody>
</table>
Patterns of gene flow

Contemporary pollen dispersal in the isolated population:

In the isolated population we applied parentage analysis to detect in a first step pollen dispersal distances in the wildlings, seed and nursery raised seedlings (Figure 2). Of the wildling samples we were able to assign 85% (N=99) to the two most likely parents at the 85% confidence level. With 83% (n=82) of the pollination events within 100 metres the pollen dispersal in the wildlings is dominated by short distance events. Four per cent (n=4) are selfing events. Further we detect three long distance pollen dispersal events between 3 km and 3.4 km from the neighbouring continuous population. The average pollen dispersal distance in the wildlings is 167m (median=39m). In the seed samples the assignment rate was 69% (N=180) of which 86% (n=154) were dispersal events within 100 metres. The selfing rate was 28% (n=51). Five seed samples were assigned to putative fathers from the continuous population at distances between 2.3 km and 3.3 km from the maternal tree. Over all assigned seed samples the average pollen dispersal distance is 139 m (median=22 m).

The parentage analysis of the nursery seedlings assigned 67% (N=133) of the samples to the two most likely parents. Eighty seven per cent (n=116) of the assigned nursery samples derived from pollination events within 100 metres and 29% (n=38) were selfing events. Four nursery seedlings were assigned to fathers from the neighbouring continuous population with distances between 2.4 km and 3.2 km. In the nursery seedlings the average pollen dispersal distance is 138 m (median =21 m).

Figure 2: Frequency distribution of pollen dispersal distances in the isolated Vateria indica population obtained from parentage analysis from different offspring life stages.
The dispersal patterns between the three different sample types primarily differ by the elevated selfing rate observed in the seed and the nursery samples compared to the wildlings. Under the assumption that we have detected all selfing events, all unassigned seed should be from outcrossing events. Based on this assumption we can estimate a minimum selfing rate as 3% in the wildlings, 20% in the seed and 19% in the nursery samples.

<table>
<thead>
<tr>
<th>Past patterns of pollen dispersal in the isolated population:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parentage assignments of small adult trees within the small isolated population assigned 9 of the 12 smaller trees to two most likely larger adults within the forest patch. The average pollen dispersal distance was 141 metres (median=53 m) which is (given the small sample size) similar to the pollen dispersal distances estimated from the three different juvenile samples in this population.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Contemporary pollen dispersal in the continuous population:</th>
</tr>
</thead>
<tbody>
<tr>
<td>At the river population we could assign 41% (N=49) of the wildlings at the 85% confidence level. Twenty two of the assigned samples were from selfed wildlings of which 21 were collected under two mother trees isolated from the next conspecifics by 60 m and 70 m. These selfing events result in a 45% selfing rate of the assigned samples. Assuming that we have assigned every selfing event we can estimate the minimum selfing rate within our samples to be at least 18% (22 out of 119 samples) (even if we missed some selfed wildlings derived from trees we did not sample). The average pollen dispersal distance was 158 m (median= 17 m) with a maximum of 2167 m with no pollen inflow from the isolated population. Because these selfing events might be exceptional and because of the low assignment rate it is doubtful if we captured a representative sample of pollen dispersal in this continuous population.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Patterns of seed dispersal in the isolated and the continuous population:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed dispersal was almost the same in all four sampling schemes with average seed dispersal distances ranging from 14m to 19m (median 5-8 m). Between 87% (156 seed samples) and 96% (128 nursery samples) of the seed were dispersed within 40 m. It is noteworthy that the seed dispersal distance derived from the nine parentage assignments of adult trees within the isolated population was with an average of 60 metres (median=20 m) considerably larger.</td>
</tr>
</tbody>
</table>

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<tr>
<th>Indirect estimates of historic gene flow:</th>
</tr>
</thead>
<tbody>
<tr>
<td>The indirect estimate of gene dispersal distances (σ) computed with SPAGeDI 1.3a (Vekemans and Hardy 2004) ranges from 23 meters and increase with decreasing effective density up to 59 meters. Based on two times the standard error the gene dispersal estimates are unlikely to differ significantly between the two contrasting adult populations (Table 3).</td>
</tr>
</tbody>
</table>
Table 3: Gene dispersal sigma (σ) in metres of Vataire indica computed in SPAGeDi 1.3a (Hardy and Vekemans 2002) DN: assumed historic density of 104 V. indica trees based on Pascal and Pelissier (1996), SE= standard error by jack-knifing over loci. SIP: small isolated population, LCP: large continuous population

<table>
<thead>
<tr>
<th>Assumed historic effective density</th>
<th>Adults SIP</th>
<th>Adults LCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_n/2$ (52 trees/ha)</td>
<td>25.2 (SE=4.2)</td>
<td>22.5 (SE=1.9)</td>
</tr>
<tr>
<td>$D_n/10$ (10.4 trees/ha)</td>
<td>58.8 (SE=9.0)</td>
<td>58.7 (SE=7.6)</td>
</tr>
</tbody>
</table>

**Patterns of fine scale spatial genetic structure**

In all cohorts we observed significant patterns of FSGS which do not differ in their decrease of kinship over the ln distance as indicated by the $Sp$-statistic. The intensity of the FSGS quantified by the $Sp$-statistic shows no significant difference when comparing the adults from the continuous population with the adults or the wildlings from the isolated population (Table 4). Despite the similar intensity of FSGS the kinship of the adults and wildlings from the isolated population is significantly different to zero only up to 40m whereas the kinship of the adults from the continuous population is significantly different from zero up to 160m (Figure 3). Indeed, the single class heterogeneity test indicates that the adults from the continuous population has a significantly higher relatedness at the 40m 80m and 160 metres distance class (p<0.01).

Table 4: Slope of the kinship coefficient over the ln distance ($b_{ld}$) and $Sp$-statistic to compare the intensity of the FSGS between the adults and wildlings from a small isolated population (SIP) as well as adults from a large continuous population LCP in Vataire indica calculated over 10m, 20m, 40m, 80m, 160m and 640m as end points of the distance class intervals.

<table>
<thead>
<tr>
<th></th>
<th>$b_{ld}$ (Standard error)</th>
<th>$Sp$ (Standard error)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vataire indica</em> adults SIP</td>
<td>$b_{ld}$=-0.027 (0.005)</td>
<td>0.027 (0.005)</td>
</tr>
<tr>
<td><em>Vataire indica</em> wildlings SIP</td>
<td>$b_{ld}$=-0.025 (0.004)</td>
<td>0.028 (0.004)</td>
</tr>
<tr>
<td><em>Vataire indica</em> adults LCP</td>
<td>$b_{ld}$=-0.023 (0.003)</td>
<td>0.026 (0.003)</td>
</tr>
</tbody>
</table>
The re-calculation of the FSGS based on different distance intervals reveals that the adult V. indica populations have a consistently more intense FSGS than the three dipterocarps from single large continuous populations investigated by Kettle et al. (2011). Contrastingly the extremely rare and fragmented Vateriopsis sechellarum has a much more intense FSGS than what we observe in V. indica (Table 5).

| Performance of nursery raised seedlings |

Of the 320 initially planted seed 260 germinated of which 202 survived for 21 months. Of these 199 were successfully genotyped. Based on the parentage analysis we split the 133 assigned nursery seedlings into selfed (n=38) and outbred individuals (n=95) to investigate if their performance differs. Of the non-selfed progeny we split these individuals according to the median kinship coefficients of the parent pairs (Loiselle et al. 1995). This resulted in the outbred nursery seedlings in a group of 48 individuals with a low kinship of parent pairs (<0.1) and 46 individuals with a high kinship of parent pairs (>0.1, one individual from this group was excluded from this analysis because the size measurement was missing). As indicated by the ANOVA there was significant variation ($F_{2,129}=6.85$, $p=0.0015$) in size among the three groups. The mean height after 21 months of growth in the selfed individuals was 31 cm, which in a Tukey HSD test differed significantly from the average size (44 cm) of outbred individuals with a low kinship of parent pairs ($p=0.001$) and from the average size (40 cm) of outbred individuals with a high kinship of parent pairs ($p=0.03$). The two groups of outbred nursery seedlings did not differ significantly ($p=0.56$) (Figure 4).
Figure 3: Fine-scale spatial genetic structure of Vatairea indica for the adult cohort of a continuous population, the adult cohort and the wildling cohort of an isolated population. The distance classes are: 10m, 20m, 40m, 80m, 160m and 640m.

Figure 4: Mean size of nursery raised Vatairea indica after 21 months of growth grouped by different levels of inbreeding estimated from the kinship of parent pairs. Error bars are 2x standard error. Differing letters (a,b) indicate significant differences between means of groups at the 95% confidence levels (p<0.05) based on a Tukey HSD test.
DISCUSSION

To sustain high levels of biodiversity in complex agro-forest landscapes, constituent tree species and their underlying genetic resources must be maintained. Our study indicates that in a small isolated and larger continuous population of *V. indica* genetic resources are effectively conserved despite significant fragmentation. Our detailed investigation of contemporary gene flow suggests that although the small isolated population is genetically insular it is sufficiently large to prevent genetic erosion. The parentage analysis of seed and nursery reared seedlings indicate relatively frequent selfing in *Vateria indica*. These selfed progeny exhibit poorer performances under common garden conditions, and selection favours outbred progeny under forest conditions. These findings add to our understanding of how tree species genetic diversity can be retained in human dominated landscapes. Based on our results we make management recommendations specifically relevant to the conservation of genetic resources in the context of restoration.

<table>
<thead>
<tr>
<th>Does a small isolated population retain comparable levels of genetic diversity to a larger more continuous population?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Our estimates of genetic diversity demonstrate no loss of genetic diversity from the adult to the wildling stage in the small isolated or the larger more continuous population. This suggests that most trees contribute to the next generation. Indeed, 73 parents of the 85 trees in the isolated population were detected in the parentage analysis of all the offspring samples. This is contradictory to our expectations and to many empirical studies comparing adults and wildlings of fragmented tree populations which show lower genetic diversity in the juveniles (e.g. Kettle et al. 2007; Sebbenn et al. 2011; Finger et al. 2012). Within the isolated population we detected significantly lower genetic diversity than in the larger population. This might be due to an initial sampling effect associated with fragmentation which retained a lower proportion of the species level diversity (discussed below).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Do patterns of gene flow and consequently genetic structure differ across life stages and between continuous and isolated populations?</th>
</tr>
</thead>
</table>
| **Contemporary pollen dispersal in a small isolated population:** Comparing the pollen dispersal pattern of the wildlings with the seed samples and the nursery reared progeny, demonstrates that pollen is dispersed predominantly within 100 metres (Figure 2). Interestingly we detect in all offspring samples between 2% and 3% pollen inflow from the nearest larger population. Although rare, these long distance pollen dispersal events contribute to the maintenance of the genetic diversity. Some of the unassigned samples might
also result from such long distance pollen dispersal events which would increase the estimated average pollen dispersal distance.

The main difference between the pollen dispersal estimates for the three offspring groups is the considerably lower selfing rate of only 4% in the wildlings compared to the selfing rate estimated for the seed samples and the nursery samples. This elevated selfing causes an excess of homozygotes which is reflected in the higher inbreeding coefficients ($F_{is}$) and the significant different $F_{is}$- values in the seed and nursery seedlings compared to the adults and the wildlings of the isolated population (Table 1 and Table 2). Because the seed, wildlings and nursery reared seedlings collected from the isolated population result from the same flowering season we assume that the initial selfing rate at fertilization was the same. The most likely explanation for the lower selfing rate in the wildlings is that selfed seed had higher mortality than outbred seed, which indicates an efficient selection against selfed progeny. The reduced selfing rate detected in wildlings causes the higher average pollen dispersal distances compared to the seed and the nursery seedlings.

**Historic patterns of realised pollen dispersal in the small isolated population**

The assignment of nine out of twelve trees with a DBH <50 cm to two parents within the grove is in line with the dominance of short distance pollen dispersal. Although the sample size of this parentage analysis within the adult trees is small, the rather high assignment rate indicates that this forest patch is isolated already for a long time period.

**Contemporary pollen dispersal in the large continuous population**

Similar to the isolated population we detect in the wildlings from the continuous population some occasional long distance pollen dispersal events confirming the potential for long distance pollen dispersal. Of the three events beyond 500 metres two are beyond 2 km, but we did not detect a single father from the continuous population. Such rare but consistent long distance pollen dispersal beyond 500 metres is similar to previously published pollen dispersal estimates made for *Dipterocarpus tempehes*, a dipterocarp species which is commonly pollinated by the giant honey bee *Apis dorsata* (Kenta et al. 2004). Indeed, 5% of the foraging distances of *Apis dorsata* are beyond 2.5 km with maximum flying distances between 13.2 and 21.8 km (Dyer and Seeley 1991). Further we detect a high selfing rate with 22 of the 49 assigned wildlings being selfed. Twenty-one of these 22 selfing events are from wildlings collected below two isolated trees (isolated by 60 and 70 meters from conspecifics). Such increased selfing in a predominantly outcrossing plant species might be a strategy to assure reproducti-
on when mating partners are lacking (Kalisz et al. 2004; Eckert et al. 2010). Comparing these selfing rates with the selfing rates of the wildlings from the continuous population is not meaningful, as these seedlings were presumably only one year old and it is questionable if they would have survived another year.

Patterns of seed dispersal in the isolated and the continuous population

Estimates of seed dispersal are comparable across all the progeny samples from both populations with average seed dispersal not exceeding 19 metres, supporting the view that seed dispersal is entirely by gravity. Still it is unexpected that between 4% and 13% of the seed are dispersed beyond 40 metres indicating that there is some occasional secondary seed dispersal. This secondary dispersal is possibly by rodents as we have occasionally observed teeth marks on seed coats (Ismail pers. obs. 2008 and 2010), secondary seed dispersal by forest rats has also been reported before in other dipterocarp species (Wells and Bagchi 2005).

Indirect estimates of historic gene flow

Because the $Sp$-statistic and the slope of the decline of kinship with the ln distance of the two adult populations are almost the same, we expect similar historic gene flow patterns. Indeed the indirect estimates of historic gene flow indicate that historic levels of gene flow estimated from the two adult populations do not differ (Table 3). This indicates that the isolated population most likely is a remnant forest patch and not a result of a founder effect which would result in more intense kinship structure and a much greater differentiation among the study populations under extreme founder events (see Finger et al. 2012). The heterogeneity test shows significantly higher relatedness at the river population at 40m, 80m and 160 meters, indicating isolation by distance patterns over longer distances.

Recommendations for the conservation and management of genetic resources in Vateria indica across agro-forest landscapes

By comparing a continuous population with an isolated population of Vateria indica our study offers insights in how genetic resources of V. indica can be managed for restoration of degraded wet evergreen forest of the Western Ghats. Vateria indica has a great potential for restoration as it grows relatively fast and survives well in degraded forest (Rai 1990). Moreover, V. indica is valuable for its timber, resin used for incense and varnish, as well as its bark, leaves and seed oil used for medicinal purposes (Shiva and Jantan 1998). Using V. indica in restoration would thus benefit biodiversity and livelihoods of local communities (Lamb 2011). Knowing at what life stage selection against inbred progeny acts is relevant for restoration management. Similarly to our findings effective selection against selfed and inbred wildlings has
been observed in other dipterocarp species (Lee et al. 2000; Naito et al. 2005). Interestingly, Ghazoul and Satake (2009) propose that selfed seeds may dilute the impact of seed predators by acting as seed predator sink, thereby increasing the survival of outcrossed seeds. In our case a similar but delayed process might act at the seedling stage. As our results demonstrate, selfed individuals are more likely to survive under benign nursery conditions than in nature. This phenomenon of elevated inbreeding under nursery conditions has been observed in other rare tree species (Kettle et al. 2008). The common early selection in tree species (Petit and Hampe 2006) might be in many species less effective when seedlings are grown in nurseries. Consequently restoration with nursery seedlings can result in the establishment of inbred populations, as demonstrated by the extreme genetic bottleneck in a planted population of V. seychellarum (Finger et al. 2012).

Transferring seed instead of nursery seedlings would ensure the natural selection against maladapted individuals and would reduce inbred progeny in restored populations. Sampling seeds for restoration over multiple flowering events will also increase the effective population size. Seeding can effectively aid forest recovery while being considerably cheaper than planting seedlings (Cole et al. 2011). We suggest therefore favouring seeding over costly nursery reared planting stock for V. indica.

Nursery reared seedlings may still be an essential part of restoration programmes for highly degraded forest, as many forest species cannot establish from seed under open conditions (Gunaratne et al. 2010). Further propagating very rare species threatened with imminent extinction can be vital for in situ and ex situ management strategies (Finger et al. 2011; Finger et al. 2012).

Alternatively wildlings could be sampled directly from the forest floor, this has two potential advantages: Firstly, progeny are more likely to be the result of several flowering events (Kettle et al. 2008). This is particularly relevant for irregularly flowering species, such as most dipterocarps from South East Asia which show sporadic synchronous mass flowering (Ashton 1988). Secondly, relocation of wildlings with soil could also transfer mycorrhizae, which have been shown to improve establishment of dipterocarps (Brearley et al. 2007; Lee et al. 2008). The downside of collecting wildlings is the risk of affecting natural regeneration and the cost implications (Kettle et al. 2008).

Our results suggest that to capture genetic diversity in seeds or wildling collections for restoration, collections should cover several km. To minimise relatedness sampling intervals should
exceed 40 metres. Ideally seed collection should ensure pairwise distances beyond 160 metres, since we know from the FSGS that beyond this distance pairs of individuals are not related more than random pairs from within the population. Similar strategies could be applied to most other dipterocarp species as they usually have limited seed dispersal and therefore often experience significant FSGS (Harata et al. 2011; Kettle et al. 2011; Finger et al. 2012). The comparison of the FSGS with the three dipterocarp species from a continuous forest in Borneo demonstrates a more intense FSGS in *V. indica*. The comparison with *Vateriopsis seychellarum* shows that the level of relatedness decreases faster with distance than in *V. indica*. As the seed dispersal is almost the same, this difference is driven by the shorter pollen dispersal distances in *V. seychellarum* which is presumably influenced by the strong fragmentation and the much smaller population sizes.

With the growing knowledge of FSGS, pollen and seed dispersal in dipterocarps, one can infer that dipterocarps with strong FSGS and limited pollen dispersal should be more vulnerable to fragmentation. Our study adds an additional aspect that has to be taken into consideration: Species with limited seed dispersal occurring naturally at high local densities can buffer deleterious effects of inbreeding by self-compatibility combined with effective selection against maladapted selfed individuals. The robustness of an isolated patch is largely determined by the distance to the next population, the pollen dispersal distance and the local population size. To find in *V. indica* at what level of fragmentation negative genetic effects have to be expected our study would have to include more replicates of populations of varying size and degree of isolation, which was in the study region not feasible due to the rarity of such patches. Therefore we cannot exclude that we detected some population-specific effects. Despite these limitations our study provides insights into essential processes which can conserve genetic variation in isolated forest fragments of a threatened dipterocarp species endemic to India. This is especially significant given the paucity of knowledge on the genetic consequences of fragmentation for dipterocarp species across their range.

| Acknowledgements |
We thank the reliable field assistants Navin H. Kumar, Monappa C.S., Chengappa S.K. and particularly Range Gowda, Harsha C. and the late Umesh who took care for the nursery. The tedious single DNA extractions conducted by Sandeep Sen and Shruthi Jayappa are esteemed a lot. Fragment analysis was conducted at the Genetic Diversity Centre (GDC) of ETH Zürich. This research was funded by ETH Zürich under the grant number ETH-22 08-2.
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CHAPTER 6

General conclusions

| Key Results |
The investigations of pollen mediated gene flow in *Dysoxylum malabaricum* using paternity analysis demonstrated that this species maintains genetic connectivity between the different forest patches at the landscape scale. The replicates of forest fragments covering a range of adult tree densities made it possible to show that pollen dispersal patterns are influenced by the local adult tree density. Trees with less than six conspecifics within 500 metres experienced an elevated frequency of short distance mating in comparison to trees in forest patches with more conspecifics. The adult trees of *D. malabaricum* show elevated kinship at short distances, as indicated by the investigations of fine-scale spatial genetic structure (FSGS). The combination of significant FSGS with increased frequency of short distance mating under low adult tree densities leads to elevated kinship among mates and genetic drift. This finding demonstrates that even tree species with great potential for long distance dispersal can experience elevated inbreeding due to fragmentation when local densities drop below a certain threshold.

To get a more complete picture of gene flow in *D. malabaricum* I estimated seed dispersal by applying parentage analysis to established seedlings. Based only on neutral nuclear markers it is impossible to assign in a parentage analysis which of the candidate parents is the pollen source or the seed source. Therefore I developed a new approach based on the simple assumption that within individual offspring pollen and seed dispersal is independent. Combining our knowledge of pollen dispersal based on paternity of seeds with parentage analysis of wild seedlings, in all but one case pollen dispersal was best explained from one patch to another when the most distal candidate parent was assigned the paternal parent. Assigning in these cases the maternity to the further parent would violate the assumption of independent pollen dispersal. Following this logic I demonstrate that seed dispersal is very limited in *D. malabaricum* and that effective migration among the forest patches of the study region is extremely rare. This finding is surprising and contrary to expectations that larger dispersers like hornbills...
should promote long distance dispersal due to their home ranges, their travelling velocity as well as their larger gut capacity and longer seed retention time (Nathan et al. 2008).

Beside reducing the number of individuals and disrupting the continuity of populations, forest fragmentation is often additionally associated with increasing anthropogenic resource use pressure which further degrades remnant forest patches by disturbances such as small scale logging, fire wood collection, grazing, hunting and invasive exotic species (Tabarelli et al. 2004). The potentially negative consequences of such multiple co-occurring stressors for species survival were emphasized already 25 years ago (Myers 1987) with increasing scientific interest in recent years (Laurance and Useche 2009). To investigate how recruitment of D. malabricum might be affected additionally to the genetic effects of fragmentation I recorded indicators of habitat degradation to assess the effects on seedling densities. The investigations on habitat degradation suggest that increased canopy openness, the abundance of pioneer species and a reduction of arboreal termite nests indicate a general reduction of habitat suitability for the recruitment of D. malabaricum. In addition I conducted a nursery experiment which demonstrated that elevated mating between related individuals directly affects seedling vigour. I interpret these co-occurring effects on seedling abundance and performance as a sign that fragmentation and habitat patch degradation both affect recruitment of D. malabaricum via genetic as well as biotic and abiotic processes.

In a second study species, Vateria indica, I compared genetic diversity, inbreeding coefficients, FSGS and gene flow in an isolated population with a continuous population. The finding that established seedlings collected from the isolated population have lower inbreeding coefficients and reduced selfing rates than seed and nursery raised seedlings, suggest that V. indica has under natural conditions an effective and early acting selection against selfed individuals. In combination with occasional realized long distance pollen flow into the isolated population these processes might reduce inbreeding and ensure the maintenance of genetic diversity of the isolated population. Further the investigations of growth performance in the nursery raised seedlings confirmed the reduced performance of selfed seedlings.

When comparing the two V. indica populations I found a reduced genetic diversity in the isolated population most likely due to some historic sampling effect caused by fragmentation. The analysis of historic gene flow and FSGS showed not much difference between the two populations of V. indica. As FSGS is a potentially important indicator for tree species susceptibility to fragmentation and because FSGS is common in many dipterocarp species I compared the FSGS of V. indica with four other dipterocarp species. This comparison revealed that the intensity of
FSGS in three dipterocarp species from continuous forests in Borneo was consistently lower whereas the extremely rare *Vateriopsis seychellarum* has a much more intense FSGS than *V. indica*. These differences are probably a combination of seed dispersal potential, population history and the degree of fragmentation.

The detailed investigations of the genetic diversity, inbreeding, pollen dispersal, seed dispersal and recruitment allowed me to make recommendations for improved conservation management of *D. malabaricum* and *V. indica*.

**General management implications**

The estimates of seed dispersal in *D. malabaricum* and *V. indica* show no realistic potential to re-colonize habitats were these species have previously been logged out. Therefore both species are very likely to depend on human intervention such as artificial seed dispersal or introduction of nursery raised seedlings to colonise forest patches. This may be essential to ensure the recovery of diverse forest communities, which support forest biodiversity.

The differing spatial distribution and response to inbreeding of the two study species requires distinct management implications for seed sourcing and conservation management.

Although long distance pollen dispersal in *D. malabaricum* does maintain genetic connectivity across the study area, management has to consider the elevated inbreeding and drift detected in forest patches with low adult tree densities. Therefore it is most important to ensure that local adult tree densities do not further deplete. Besides introducing *D. malabaricum* into forest patches were the species was presumably extirpated, enrichment planting or seeding in forest patches where adult trees occur at low densities should be considered. Artificial gene flow by seed exchange or transplanting nursery raised seedlings among groves would help to counteract the observed genetic differentiation at the landscape level. Seed should be sourced from the forest patches with more than six individuals as these seed show less inbreeding. Single isolated adult trees attract pollinators over long distances from a variety of different fathers and therefore offer a valuable source of genetically diverse and outbreed seeds which could also be used for forest restoration. Sampling seed from several sites with a minimal number of trees and from single isolated trees should minimize the fraction of inbred seed. In the long term the genetic connectivity at the landscape scale would be strengthened if more trees were maintained at intermediate distances (0.5 – 1 km). This would likely reduce inbreeding within the groves but would require that coffee farmers plant this species in their estates. Providing the incentive to coffee farmers to plant native shade trees is a complicated socio-economic problem, discussed later.
The central finding relevant for restoration of *Vateria indica* is that selfed individuals are more likely to survive under benign nursery conditions than in the forest floor. Advancing our understanding of selection for outbred progeny has direct relevance for restoration management. My results demonstrate that restoration with nursery raised seedlings can result in the establishment of inbred populations. This can be easily avoided by transferring seed or established seedlings instead of nursery seedlings. To ensure sampling the genetic diversity of a region, the differentiation at the landscape scale as well as the strong FSGS due to the limited seed dispersal has to be taken into account. The landscape scale genetic differentiation and the estimates of seed dispersal indicate that sampling from more than one population and over distance intervals of around 40 metres will maximise genetic diversity while minimising sampling of half-sibs.

**Tree species susceptibility to habitat fragmentation:**

By placing the findings of this thesis in the context of other studies on responses of tree species to fragmentation I discuss general aspects of tree susceptibility to fragmentation. It seems plausible that the responses of different tree species to fragmentation not only depend on species specific traits but also depend on the investigated spatial arrangement of the adult trees and respectively the degree of spatial isolation. Therefore I emphasize that generalizations on tree species limited vulnerability to genetic fragmentation should to be made cautiously.

**Inbreeding in tree species**

Trees produce over their lifetime a vast number of offspring of which only a small fraction will eventually survive to maturity. This provides considerable selection against maladapted and inbred individuals during early life stages favouring local adaptation (Petit and Hampe 2006). This extreme selection pressure is in tropical tree species often associated with relative stable environmental conditions. If these conditions are changed by forest fragmentation, recruitment and survival rates might change which may have major implications for the population dynamics of a species. Because tree species can endure periods of adverse conditions for reproduction without major demographic consequences (Petit and Hampe 2006) the consequences of changed population dynamics for species persistence are difficult to investigate (see “Inbreeding in tree species and long term persistence” below). Elevated selection against inbred juveniles due to forest fragmentation may not implicitly threaten species persistence because these may not be significantly beyond natural background selection in trees. The production of inbred in *V. indica* might be a strategy to dilute the effects of herbivores as suggested by Ghazoul and Satake (2009) for inbred seed. This questions the common assumption that inbreeding depression as such is negative for populations. It has to be kept in mind that
inbreeding depression has only negative consequences if it effectively reduces the likelihood of individuals reaching reproductive age. In *D. malabaricum* this might be the case as indicated by the lack of small size classes.

**Characteristics of pollen and seed dispersal**

Seed dispersal in tree species is the initial process that shapes the spatial and genetic structure of the adult population, at least in their favoured habitat. Limited seed dispersal was shown to increase spatial aggregation of many tropical adult trees (Condit *et al.* 2000). This link between spatial arrangement of adult trees and the seed dispersal potential presupposes an evolutionary link between seed dispersal and pollen dispersal strategy. If a tree species has evolved to occur naturally at low densities, pollination must have co-evolved to ensure that the sparsely distributed trees receive pollen to ensure reproduction. On the other hand, species with very limited seed dispersal which occur naturally at high densities do not require reliably pollen dispersal to conspecifics over long distances. Indeed, it has been suggested that a positive correlation between pollen and seed dispersal distances should be common in tropical tree species (Hardy *et al.* 2006). When the spatial arrangement of trees is altered by human disturbance the response of a tree species depends largely on how robust the dispersal of pollen and seed is to maintain genetic connectivity. Seed dispersal by animals and wind is commonly reduced by habitat fragmentation (McConkey *et al.* 2012) with tree species depending on larger vertebrates expected to be most sensitive (Cramer *et al.* 2007). Similarly, theory predicts that susceptibility of pollen dispersal to changing habitats is thought to be more vulnerable the more specialised the pollinator is (Bond 1994) whereas empirical evidence for such a difference is lacking (Aguilar *et al.* 2006).

**The role of mating system**

The investigations on pollen and seed dispersal conducted in this thesis revealed that *D. malabaricum* is predominantly outcrossing while *V. indica* is a mixed mating species. Based on my findings I cannot be sure how consistent this mixed mating is in *V. indica*. The selfing rate of other tropical tree species with mixed mating was shown to depend on the adult tree density (Obayashi *et al.* 2002; Fuchs *et al.* 2003) and to vary across years (Murawski and Hamrick 1992b; Lobo *et al.* 2013), populations (Ward *et al.* 2005; Breed *et al.* 2012) and individuals (Murawski and Hamrick 1992a). Increased selfing due to spatial isolation in predominantly outcrossing plants is thought to be a reproductive assurance strategy in the absence of pollinators or mating partners (Eckert *et al.* 2010) which might be an important trait of species adapted to colonize new habitats (Levin 2010). Outcrossing plant species generally show less intense FSGS than self-compatible species (Vekemans and Hardy 2004) which could be driven
by selfed individuals but could also indicate more frequent mating among related individuals. There is theoretical and empirical support that inbreeding depression in plants tends to decrease with an increase in selfing rate due to more effective purging of deleterious alleles (Husband and Schemske 1996; Byers and Waller 1999). These differences between self-compatible and outcrossing plant species are likely to be relevant for tree species susceptibility to fragmentation. Predominantly outcrossing species like *D. malabaricum* may not be adapted to high levels of inbreeding and may therefore suffer more from increased bi-parental inbreeding caused by fragmentation. In support to this idea, it was shown that fragmentation affects outcrossing plant species more than self-compatible species by reducing their genetic diversity (Honnay and Jacquemyn 2007; Ng *et al.* 2009) and their reproductive output (Aguilar *et al.* 2006). In *D. malabaricum* the loss of genetic diversity is to some degree counterbalanced by the extensive pollen flow, but I also show that further reduction of the already small groups of trees could reduce gene flow which results in deleterious genetic consequences. Therefore it seems that reduced gene flow by anthropogenic disturbance affects outcrossing species more severely, especially if their gene flow is already limited. Such limited gene flow could be inferred from the FSGS and should not be inferred from the assumed seed dispersal potential of the vector, because this can be misleading as shown for *D. malabricum*.

<table>
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<th>The benefits of native trees in agricultural systems</th>
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| My thesis suggests that gene flow across the landscape could be promoted in *D. malabarism* if more trees would be present within the intervening agricultural matrix. Therefore it is of great importance to consider the potential benefits of trees in agricultural systems. Beside the central role of trees in sustaining biodiversity in agricultural landscapes (Tews *et al.* 2004; Manning *et al.* 2006; Fischer *et al.* 2010), agroforestry provides a multitude of additional benefits. Shade trees are maintained to ameliorate physiological stress which enhances longevity of perennial crops like coffee and cocoa (Beer *et al.* 1998). Further, there is growing scientific interest in agro-forestry demonstrating a multitude of ecosystem services and environmental benefits of shade trees. In particular such agro-forest systems improve soil fertility, improve air and water quality, enhance drought resistance, provide biological pest control and substantially sequester carbon (Jose 2009; Tscharntke *et al.* 2011). Trees in agro-forest systems can contribute substantially to household income by providing fuel wood, timber and non-timber forest products such as fruits (Rice 2008; 2011). Trees in the agricultural matrix can also benefit agricultural production, as wind brakes (Jose 2009). Economic incentives to maintain or adopt agro-forestry might be provided by payments for ecosystem services and certification schemes (Tscharntke *et al.* 2011) but also depend greatly on markets for agroforestry tree products (Leakey *et al.* 2005). Despite the multitude of potential benefits of maintaining a diverse
community of trees with agricultural landscapes there is a global trend in intensification and removal of shade trees in traditional shade tree production systems due to short term monetary benefits of yield increase (Tscharntke et al. 2011 and references therein). Evaluating the tradeoffs perhaps requires a more balanced evaluation of the positive and (largely ignored) negative costs related to trees, including branch and tree falls, hosting pests, allelopathic effects, competition for growth resources (Beer 1987), lower coffee quality (Bosselmann et al. 2009) and clogging of flowers by litter (personal observation).

| The potential for *Dysoxylum malabaricum* and *Vateria indica* in the agricultural landscape of Kodagu
The valuable genetic resources represented in the tree species of the diverse landscape of Kodagu are gradually lost because the trees are cut or not replaced when they die. Although I demonstrate that it would be beneficial for the tree species to be planted in sacred groves and coffee plantations there are significant socio-economic obstacles that have to be considered.

| Sacred groves
Planting native trees in sacred groves is to my knowledge currently not considered although they provide a great potential to harbour rare species and support diversity at the landscape scale (Bhagwat et al. 2005). Sacred groves of Kodagu are being degraded and reduced in extent due to cultural changes (Chandrakanth et al. 2004; Ormsby and Bhagwat 2010). The village temple committees who are in charge for the management of the sacred groves focus on temple maintenance and organisation of festivals. This goes along with clearing the forest around the temples creating a big gap in the forest (Garcia C.A. and Pascal J.P. 2006). This development facilitates that the spiritual focus shifts away from the forest to the temple itself. The forest seems mainly of interest when temple committees request permission to cut trees from the forest department, which is generally granted when the timber or its revenue is used for temple construction. Before *D. malabaricum* became very rare it was regularly used for temple construction. This is also reflected by the local species name “devadaru” where the prefix “deva” refers to a god or goddess. Under these circumstances the forest department should not only permit cutting of trees but should instead get temple committees involved in planting valuable and rare hard wood species. Such engagement of the forest department with the temple committees would raise awareness among temple committees and foster better management of the natural resources of the sacred groves. Given this context *D. malabaricum* can serve as a key species as it has not only a high economic value but also a spiritual value. The incentive to plant *V. indica* in the scared groves is probably less because its timber and its non-timber forest products are rarely used in Kodagu. As *V. indica* was shown to be suitable for
restoration of wet evergreen forests (Rai 1990), this species should be considered as a valuable species for planting in the most degraded sacred groves and in the degraded private forest where the canopy trees are completely gone.

Coffee plantations

In the coffee plantations the maintenance of native trees is mainly a question of shade management and tree rights (Garcia et al. 2010). Tree rights in Kodagu can vary from estate to estate but in general, farmers must obtain permits to fell, transport and sell native trees for timber. The lack of timber rights to trees on their own land coupled with complex bureaucratic procedures cause administrative costs as well as corruption, bribing and the promotion of an illegal timber market (Garcia et al. 2010). No such regulations exist for exotic fast rotation timber such as *Gervillea robusta*, which makes this species the most commonly planted shade tree in Kodagu (Garcia et al. 2010). The investigated tree species *V. indica* and *D. malabaricum* represent in this respect interesting case studies:

*Dysoxylum malabaricum* might be a suitable shade tree due to its apical strait growth, the late branching, the rather light crown that produces speckled shade, the high timber value and the rough bark ideal to support pepper vines. Indeed, I distributed 250 seedlings of *D. malabaricum* to temple committees and coffee farmers in May 2011. This event was well attended and my nursery stock could certainly not meet the demand, indicating that there is potential to promote native trees in coffee estates. Therefore the loss of native trees might be also influenced by the availability of seedlings from different species, which should be recognised by private and state nurseries which predominately provide *G. robusta* seedlings.

In contrast *V. indica* has a much lower timber value, extensive roots, strong branching, dense foliage and a smooth bark. Therefore this species is unpopular in coffee estates. Some potential benefits within the agricultural landscape exist: One farmer mentioned to me the large amount of leaf litter that is produced by *V. indica* trees at the border of his estate which he uses for mulching. Another framer requested 40 seedlings from my nursery experiment for establishing a wind break. It is also apparent that *V. indica* stabilizes the river beds and thereby provides important protection to the bordering coffee plantations. This species also has a considerable amenity value which is reflected by extensive alleys with *V. indica* planted along the roads in the north of Karnataka. Therefore *V. indica* has a potential to be planted at the borders of coffee plantations and could be useful for soil erosion control.
Future work

Suitability of native trees for shading

Concerning the complex landscape mosaic in Kodagu the eminent question is how to maintain the depleting native tree diversity across the landscape, especially the native shade trees in the coffee estates and the sacred grove forests. Although this thesis provides important information on potential responses of tree species to spatial isolation and its management implications, the maintenance of trees in agricultural landscapes is in addition to an ecological question also an agro-economic question. One major understudied aspect is the identification of native tree species which have properties appreciated by farmers. Beer (1987) compiled some desirable characteristics for perennial crop shade trees which in summary are: Minimal competition with the crop for water, nutrients and below as well as above ground space. Further shade trees should have a deep rooting system to avoid tree fall, a strait fast un-forked growth, a light crown and provide valuable wood or fruit. In areas like Kodagu where pepper is an important intercrop a rough bark is preferred to support the vines. Given the growth form and the valuable timber *D. malabaricum* meets several attributes of a favourable shade tree and therefore it would be interesting to investigate the growth rates and its effects on nutrient and water competition with coffee bushes. More generally there is a gap in our knowledge about which native trees best meet the characteristics of shade trees, especially concerning their growth rate. These growth rates might be very different in coffee plantations than in natural forest due to more light, more space and better nutrient supply.

Inbreeding in tree species and long term persistence

A major understudied ecological question for tree species persistence in fragmented landscapes is the impact of inbreeding on population dynamics and its implications for long term persistence. Because inbreeding depression might be an inherent strategy to select against inbred and maladapted juveniles, inbreeding depression in trees should only be interpreted as a threat when it really reduces the amount of individuals reaching reproductive age or the fitness of mature inbred trees. Ideally, the long term fitness consequences of inbreeding depression due to fragmentation would be investigated by tracking individuals from germination to maturity which is in trees due to the long period till maturity remains very difficult. The advances of dendrochronology in the tropics have an often underestimated potential to investigate such long term population dynamics (Worbes 2002). With respect to the rather clear tree rings and the observed lack of small sized trees of *D. malabaricum*, a dendrochronological study might help to validate our interpretation of insufficient recruitment from the juvenile to the adult stage. It would be especially interesting to age trees and investigate changes in gene flow across different age classes and if these changes correlate with the abundance of trees.
This would be preferably done in a species which has no timber value and therefore the age structure would not be skewed by selective logging.

**Purging of deleterious alleles in tree populations**

Strong selection can purge deleterious alleles in plant species but is an inconsistent force across taxa (Byers and Waller 1999). Under which conditions such purging can act is difficult to investigate with long lived organisms like trees. It would be possible to investigate such processes in tree plantations which were established a long time ago and where the planting stock was collected from one isolated mother tree only and where all seed are selfed (e.g. Finger et al. 2012). Investigating whether such inbred plantations produce few superior individuals would confirm the potential of a tree species to purge deleterious alleles (at the cost of genetic variability).

**Seed dispersal in continuous habitats**

Comparing the seed dispersal patterns of *D. malabaricum* from this thesis with seed dispersal patterns in continuous populations would be useful. It seems likely that in continuous populations the movements of the Malabar Grey hornbills (*Ocyceros griseus*) might change and that a variety of dispersers such as imperial pigeons (*Ducula* sp.) and the Great Pied hornbill (*Buceros bicornis*) could enhance seed dispersal distances. Comparing the seed dispersal distances, the age structure, the seedling densities and seedling vigour with the fragmented population would better reveal the implications of fragmentation on seed dispersal in this species.

**The importance of FSGS and mating system for tree species susceptibility to fragmentation**

Considering the potential of the mating system and levels of FSGS as predictors of a tree species susceptibility to fragmentation (discussed above) it might be a valuable scientific contribution to investigate this rather theoretic expectation. With the growing number of studies investigating the effects of fragmentation on FSGS it should be soon possible to quantitatively assess the results with a meta-analysis. This would allow to test whether reproduction of plant species is differentially susceptible to habitat fragmentation depending on FSGS in combination with contrasting self-compatibilities.

**Interactions of fragmentation and habitat degradation**

The investigations of the effects of habitat degradation and fragmentation show that genetic and ecological effects co-occur. What remains unclear is if genetic effects of fragmentation and reduced seedling survival due to adverse habitat conditions act additively or synergistically. Ho-
However, my investigations on seedling densities provide a valuable baseline study to investigate direct causal effects of fragmentation and degradation that affect species survival. Based on my results it seems most promising to investigate the effect of reduced air and soil moisture due to canopy gaps on seedling survival. This would have to be done by tagging seedlings to track their survival under contrasting canopy openness. To investigate direct interactions with inbreeding depression it would be important to collect tissue samples for genotyping short after germination.

| Synthesis |
Successful reproduction is essential to ensure species persistence. By investigating pollination and seed dispersal this thesis investigates processes core to reproductive ecology and conservation biology of tree species. In particular it was shown that the potential for long distance pollen dispersal not necessarily precludes inbreeding. In both species inbreeding affected seedling vigour under controlled conditions, which in the case of *D. malabaricum* was directly linked to the local density of adult trees. For *V. indica* my results suggest that very isolated forest patches can be resilient to genetic erosion and inbreeding partly due to effective selection against selfed offspring and occasional long distance dispersal. Another important finding is that in both species the expected seed dispersal based on the vector (passive vs. large bird) can be very misleading. Knowing these potential responses of trees contributes to our understanding of the diverse species specific responses that tree species might have to fragmentation. My study provides a baseline for improved management that avoids genetic erosion of important tree species in a complex agro-forest landscape and thus could facilitate persistence of these species in human modified landscapes. The management recommendations I make would certainly aid short and long term survival of these species and point to important considerations when using these tree species for forest restoration. Such restoration depends on suitable genetic resources which are represented by the two study species and presumably by a multitude of rare tree species occurring in the agriculturally dominated landscape of Kodagu.

Although the vital role of trees and forest structures for supporting biodiversity in human dominated landscapes is now widely recognized (Peh *et al.* 2006; Bhagwat *et al.* 2008; Perfecto and Vandermeer 2008; Anand *et al.* 2010; Mulwa *et al.* 2012) it remains unclear how well genetic diversity is maintained within such landscapes. My study contributes to our understanding of how genetic diversity of tree species in human dominated landscapes can be maintained. Because genetic diversity is the foundation for all biological diversity as well as the persistence and evolutionary potential of species (Laikre *et al.* 2010) we must endeavour to conserve this fundamental component of our natural heritage.
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Development of polymorphic microsatellite markers for the critically endangered and endemic Indian dipterocarp, *Vateria indica* L. (Dipterocarpaceae).

with A. Buser | J. Ghazoul | G. Ravikanth | R. Uma Shaanker | C.G. Kushalappa | C.J. Kettle |
Published in Conservation Genetic Resources (2012), DOI 10.1007/s12686-012-9829-9

**ABSTRACT**

*Vateria indica* (Dipterocarpaceae) is an economically and ecologically important canopy tree endemic to the Western Ghats, India. The species has undergone extensive habitat loss and overexploitation and is therefore listed as ‘critically endangered’ on the 2012 IUCN Red List. We developed ten polymorphic microsatellite loci for *Vateria indica*. In addition, we confirm cross amplification and variation in two loci isolated from the closely related but geographically disjunct species *Vateriopsis seychellarum*, previously published by Finger et al. (2010). The twelve microsatellite primers screened on 48 adult samples of *Vateria indica* had 5-11 alleles per locus (mean of 8.5 per locus) with an average polymorphic information content of 0.64 across loci. Expected heterozygosity ranged from 0.44-0.84. These markers will enable us to quantify population genetic diversity in habitat fragments and to study fine scale spatial genetic structure and contemporary gene flow.
**Vateria indica** (Dipterocarpaceae) is an economically and ecologically important canopy tree endemic to the Western Ghats, India. The species is listed as ‘critically endangered’ on the 2012 IUCN Red List because it has undergone extensive habitat loss and overexploitation (Ashton 1998). *V. indica* also provides non-timber forest products including gum resin used for incense and in traditional medicine for its anti-microbial properties (Grover and Rao 1981).

The genus *Vateria* is principally bee pollinated (Ashton 1988). Dipterocarp species generally have limited dispersal (Kettle 2012), we thus predict similarly limited dispersal of the wingless fruit of *Vateria indica*. Understanding the consequences of habitat degradation and fragmentation for genetic diversity and contemporary gene dispersal by pollen and seed in *V. indica*, will help to inform conservation. Furthermore, *Vateria indica* provides the opportunity to increase our understanding of the reproductive ecology of dipterocarp tree species in highly fragmented habitats (Kettle et al. 2012; Finger et al. 2012).

To this end we developed 10 *de novo* microsatellite markers for *V. indica*. A 454 sequencing library was generated from size selected sheared fragments of genomic DNA using standard Roche reagents. The 454 library was analyzed on a Roche 454 platform using the GS FLX titanium chemistry. The total 41’308 reads had an average length of 336 base pairs. Of these, 452 contained a microsatellite insert with a tetra- or a trinucleotide of at least 6 repeat units or a dinucleotide of at least 10 repeat units. Suitable primer design was possible in 107 reads, of which 20 were labeled with an M13-tag at its 5’-end described by Schuelke (2000) and tested for polymorphism on 15 samples. Of these 20 primers, the ten most variable loci were optimized and assembled in three multiplex PCR with fluorescent labels on the 5’end of the forward primer (Table 1). Polymorphism of these ten primers was tested with 48 *V. indica* adult tree samples collected from a single river catchment in Kodagu district, southern Karnataka, which lies within the Western Ghats biodiversity hotspot.

Genomic DNA was extracted from silica dried leaves of *V. indica* (*n* = 48) using a CTAB extraction method (Sambrook et al 1998). PCR was carried out in 10.3µl reactions with 2.06µl of 5X PCR buffer (Promega colorless Flexi GoTaq PCR buffer), 0.66µL of 25mM MgCl2, 1.03µL of 0.2mM dNTPs, 0.12µL of the 5µM labeled forward primer, 0.25µl of the 10µM unlabeled forward primer, 0.62µl of the 5µM reverse primer, 0.1µL of 5U/µL Taq polymerase (Promega), and 1.3µL DNA template (c. 10 ng). The missing volume to reach 10.3 µl was adjusted for each of the 3 multiplex PCR. PCR were carried out in a Bio-Rad Dyad Cycler with the following cycling conditions: an initial denaturation of 95°C for 5 min followed by 30 cycles of 94°C for 30 sec, primer-specific temperature (56°C for multiplex reaction 1 and 2 or 52°C for multiplex reaction
3) for 1 min and 72 °C for 1 min. The reaction was ended with a final extension time of 72°C for 5 min. We used an ABI3730 for fragment analysis and Genemapper 3.5 software (Applied Biosystems) to score the fragments relative to a LIZ 500 size standard.

Descriptive statistics (number of alleles, observed and expected heterozygosities) were generated using GenAlEx 6.4 (Peakall and Smouse 2006). The polymorphism information content (PIC) and deviations from Hardy–Weinberg equilibrium was calculated in Cervus 3.0 (Marshall et al. 1998). Linkage disequilibrium was tested using GENEPOP (Raymond and Rousset 1995) and the resulting P-values were adjusted with a sequential Bonferroni correction described in Rice (1989). All twelve loci were polymorphic with 5–11 alleles and a total number of 87 alleles detected over all samples. Observed heterozygosity values ranged from 0.42 to 0.88 and expected heterozygosity from 0.44 to 0.84. There was no evidence for scoring error due to stuttering or due to large allele dropout and no evidence for the presence of null alleles according to MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004). No significant deviation from Hardy–Weinberg equilibrium was detected in any loci. The test for linkage disequilibrium revealed some association between loci Vi18 and Vi55 and between Vi55 and Vi60 (p<0.05). Two primers isolated from the closely related Seychelles endemic dipterocarp Vateriopsis seychellarellum (Finger et al. 2010) also provide useful molecular markers for V. indica (see table 1). These 12 primers will provide a valuable tool for studying the genetic diversity and reproductive ecology in this important Indian tree species.
Table 1: Characteristics of twelve microsatellite loci in *Vateria indica*; Abbreviations: F: forward primer; R: reverse primer; Ta: annealing temperature; A: number of alleles; Ho: observed heterozygosity; He: expected heterozygosity; PIC: Polymorphic information content; fluorescent labels are specified in italic font at the 5’ end of the respective forward primer. 48 individuals were analysed for each locus in *Vateria indica*. *See Finger et al 2010 for full details.*

<table>
<thead>
<tr>
<th>Locus</th>
<th>GenBank Accession no.</th>
<th>Multiplex reaction</th>
<th>Primer sequence (5’-3’)</th>
<th>Repeat motif</th>
<th>Size range (bp)</th>
<th>Ta (°C)</th>
<th>A</th>
<th>Ho</th>
<th>He</th>
<th>PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>V93</td>
<td>KC146392</td>
<td>1</td>
<td>F: FAM-GGGGTTCAGATGGGATGCGAG&lt;br&gt;R: ATGAGAACAAGGCGAATG6C</td>
<td>(AT)$_{19}$</td>
<td>79-95</td>
<td>56</td>
<td>10</td>
<td>0.771</td>
<td>0.736</td>
<td>0.706</td>
</tr>
<tr>
<td>V15</td>
<td>KC146393</td>
<td>1</td>
<td>F: ATTO585- ATGGGAGGGCGCTGACGATG&lt;br&gt;R: ACTAGTCAAACCTGTCTTGCG</td>
<td>(AT)$_{24}$</td>
<td>119-143</td>
<td>56</td>
<td>11</td>
<td>0.771</td>
<td>0.836</td>
<td>0.822</td>
</tr>
<tr>
<td>V16</td>
<td>KC146394</td>
<td>1</td>
<td>F: ATTO532-AGAGCTGTTAGCACAGAGAACTAAC&lt;br&gt;R: AGTGGAGCTATGCAAGGAG</td>
<td>(AT)$_{20}$</td>
<td>187-199</td>
<td>56</td>
<td>6</td>
<td>0.646</td>
<td>0.594</td>
<td>0.563</td>
</tr>
<tr>
<td>V55</td>
<td>KC146395</td>
<td>1</td>
<td>F: ATTO550-AGATCAGAGGAGCAGCAGG&lt;br&gt;R: TGATCTTGGGAGAAGATGTTTT</td>
<td>(AATA)$_{2}$</td>
<td>198-254</td>
<td>56</td>
<td>10</td>
<td>0.688</td>
<td>0.709</td>
<td>0.675</td>
</tr>
<tr>
<td>V45</td>
<td>KC146396</td>
<td>1</td>
<td>F: ATTO550-TGGTTGTCCTTCTTCGTGAG&lt;br&gt;R: TGACCTTGGGGAGAAGATGTTTT</td>
<td>(TA)$_{2}$</td>
<td>151-183</td>
<td></td>
<td></td>
<td>0.417</td>
<td>0.441</td>
<td>0.398</td>
</tr>
<tr>
<td>V13</td>
<td>KC146397</td>
<td>2</td>
<td>F: ATTO550- TAGACAGAGGAGCAGCAGG&lt;br&gt;R: TGTTAATCGCTACATGATGCC</td>
<td>(TT)$_{2}$</td>
<td>124-145</td>
<td>56</td>
<td>6</td>
<td>0.604</td>
<td>0.637</td>
<td>0.599</td>
</tr>
<tr>
<td>V60</td>
<td>KC146398</td>
<td>2</td>
<td>F: FAM-CATGAATATGCGATTATG&lt;br&gt;R: AACTCTGTCCTGCCTCAACG</td>
<td>(ATT)$_{2}$</td>
<td>211-229</td>
<td>56</td>
<td>6</td>
<td>0.646</td>
<td>0.671</td>
<td>0.608</td>
</tr>
<tr>
<td>V91</td>
<td>KC146399</td>
<td>2</td>
<td>F: ATTO565- TGCAAGACTCACGAAATACG&lt;br&gt;R: CCGAGTGTTGAAATACCGC</td>
<td>(ATT)$_{2}$</td>
<td>227-256</td>
<td>56</td>
<td>7</td>
<td>0.66</td>
<td>0.664</td>
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<tr>
<td>V18</td>
<td>KC146400</td>
<td>3</td>
<td>F: ATTO565-AGCTCAATTTGGCTTCTTTAG&lt;br&gt;R: ACCGCTG6GCTATGAGGAG</td>
<td>(TAT)$_{15}$</td>
<td>213-249</td>
<td>52</td>
<td>6</td>
<td>0.542</td>
<td>0.595</td>
<td>0.564</td>
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<tr>
<td>V78</td>
<td>KC146401</td>
<td>3</td>
<td>F: ATTO532- TGCAGTTTGGTTGCTTCTTTG&lt;br&gt;R: AGCTCAAGTGGATGAGGCGT</td>
<td>(AAT)$_{2}$</td>
<td>232-271</td>
<td>52</td>
<td>8</td>
<td>0.875</td>
<td>0.817</td>
<td>0.793</td>
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<tr>
<td>Vat14*</td>
<td>GUS91485</td>
<td>2</td>
<td>F: ATTO32- CTCCGCTATGATG&lt;br&gt;R: ATCTATGCAACGCTTCCTAGC</td>
<td>(TC)$<em>{2}$TT(C)$</em>{5}$</td>
<td>87-97</td>
<td>56</td>
<td>5</td>
<td>0.688</td>
<td>0.672</td>
<td>0.626</td>
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<tr>
<td>Vat10*</td>
<td>GUS91482</td>
<td>3</td>
<td>F: FAM-TGCGAGAAATCGAGCCTTAG&lt;br&gt;R: CATAAACAGATGGACCTCAGC</td>
<td>(CT)$_{17}$</td>
<td>114-162</td>
<td>52</td>
<td>8</td>
<td>0.75</td>
<td>0.729</td>
<td>0.697</td>
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<td>Acknowledgements</td>
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</table>
We thank Kirsti Määttänen for ensuring that our laboratories run smoothly and for additional support. We also thank Shruthi Jayappa for the single DNA extractions. Fragment analysis was conducted at the Genetic Diversity Center of ETH Zürich. This research was funded under grant number ETH-22 08-2 ETH, Zürich.
REFERENCES


ACKNOWLEDGEMENTS

All my efforts, interest and enthusiasm would have been in vain to successfully conduct this thesis without the contribution of each and every individual whom I shall mention by name. Prior to doing so, I want to reiterate that the order in which I refer to the many supporters, does not in any way belittles their contribution towards my thesis.

First I would like to thank my supervisors Chris Kettle, Jaboury Ghazoul, C.G. Kushalappa, Ravikanth G. and R. Uma Shaanker for their unconditional support that made this thesis possible.

Chris’s contribution to this thesis cannot be underestimated: He taught me the molecular techniques, analysing molecular data, scientific writing and some diplomacy skills. At the same time, his pragmatic approach to deal with problems helped me tremendously in achieving my goals. After discussing any difficulties with Chris, I always felt better than before and if the obstacles were not negligible, they at least felt manageable. I also thank him for his continuous encouragement, for regularly visiting me in India and for always being available via Skype or e-mail especially during field work. All these stellar attributes of him classifies him as an ideal supervisor. Besides all these professional attributes I appreciate that he, besides being a mentor, has become a true friend of mine.

Without Jaboury’s support I would not have started writing the proposal for this thesis. I am also grateful for his confidence in me and letting me work in India, which was only possible due to his long term collaborations with Uma Shaanker and Kushalappa. I also thank him for commenting on my manuscripts without major delay. Although it needed some efforts from my side to get accustomed to his frank critiques it was an enriching experience to exchange views and reaching a common understanding.

I am indebted to Kushalappa’s un-bureaucratic and logistic support which was indispensable for conducting field work in Kodagu. The success of the field work in India bestowed on his shoulder. To have Uma Shaanker as co-supervisor was an additional valuable experience. His amazingly broad scientific interest and enthusiasm was inspiring and I am lucky that I could profit from his acquiescent inputs which helped me to broaden my views on ecology.

I was also very lucky that Ravikanth got involved in this project. His scientific input during several field trips and discussions is esteemed a lot. Additionally, it would have been impossible to
work efficiently in the lab, in order to find reliable lab assistance and to get all the DNA samples safely to Switzerland.

I also like to express my gratitude to the external co-referee Olivier Hardy. He agreed without any hesitation to evaluate my thesis and to attend my viva. I also wish to extend my gratitude to Alex Widmer who unhesitantly agreed to be co-referee of my thesis.

I cannot imagine how I could have finished my thesis without constant discussion and exchange with my fellow students – I could share many nice moments and with most of them also office space. This includes Janice Lee, John Garcia, Zuzana Burivalova, Ester Frei, Thomas Hahn, Philippe Matter, James Smith, Claire Tito de Morais, Julia Born, Ernest Henning as well as the post-docs Josephine Haase and Andrea Pluess – I sincerely hope that in future our paths will cross again.

My special thanks go to Aline Finger. Having my work place opposite to hers triggered a flood of discussions which helped me to understand the stashed acts of my doings. Her inputs provided me a great boost at the end of my second year and helped me tremendously to get an overview on all the different available software applications to analyse my genetic data.

One of the kindest person I ever met is Smitha Krishnan. I am grateful that I had the chance to meet her. During my field work, I knew that I could rely on her assistance in any case of trouble. She also paved the access to numerous field sites I worked. Her name was a most valuable door opener.

No success without a touch of gossip. Florian Knaus and Ankara Chen provided me with valuable insights towards the functionality of the group mechanisms. However, Ankaras’ administrative work should not be forgotten and is highly appreciated.

Special thanks are extended to Kirsti Määtänen who performs her tasks with a minimum of administrative hassle for the lab users. I enjoyed having such a cheerful and understanding lab manager.

Tania Torossi from the GDC also deserves special thanks for her professional assistance on molecular issues while being always exhilarant.

It is hard to imagine how my computer could have worked without major problems if Gilbert Gradinger would not have been there. I acknowledge his efforts and thoughtfulness on the sometimes difficult situations of PhD students as well as on the state of the world.
My field assistants Naveen Kumar, Monappa C.S., Range Gowda, Chengappa S.K. and the late Umesh deserve an acknowledgement for their support. They were the most reliable and hard-working field assistants I could imagine and at the same time made the hard field work an enjoyable experience. I also want to thank Harsha who in my absence, took diligent care for the nursery experiment and delivered accurate results.

Also indispensable were the professional skills of Mike Charkow who climbed so many trees in unfavourable conditions. The exceptional amount of trees he climbed in a relative short fruiting season adds much to the value of this study. Sláinte mhath to you Mike.

The lab assistants Sandeep Sen and Shruti Jayappa who assiduously conducted most of the single DNA extractions in India and provided with their accurate work the bases of for the successful analysis.

In this respect it is also important to esteem the valuable contribution of Sumangala who taught me CTAB extractions, helped me to perform my first successful PCR on *Dysoxylum malabaricum*. She heads the molecular lab at ATREE very professionally and supervised the lab assistants in my absence.

I also want to thank all the staff working at the SEC-Lab: They were always full of understanding towards the strange and freaky PhD student who constantly needed the 2 ml pipette. In particular, I must mention Shivuprakash, Sentil, Mohan, Manjunath, Sagita, and Rama for a very joyful time at the GKVK campus in India.

Jenu Gowda was one of the very important people during field work. Besides teaching about life in India he was always so considerate in helping with un-countable small matters while organizing life in India. Especially I would like to thank him for sharing his flat with me during my second last field season and his unconditional help making my last short but very important field trip a very fruitful as well as an unforgettable experience.

Life in Ponampet and Gonikopal would have been often very dull without the hospitable students and researchers who were also based there. I want to thank Katy Demps, Fabien Charbonier, Jeremy Vende, Philippe Vaast, Francisco Zorondo-Rodriguez and Claude Garcia - thanks once again for the great time which I had with you in Kodagu.

I also would like to thank my sandpit friend Andi Buser, who sat with me to select the most promising primers for *Vateria indica*. Without his expertise it would have been a tedious task
to get the multiplex PCR running so smoothly. His valuable contribution expedited the entire work by at least half a year.

I would also like to extend my acknowledgement to my flat mates: Eva Geilinger, Simon Degelo, and Marianne Gehring who took care for the apartment in my absence and when I was too busy to contribute towards the housekeeping. In particular, I want to thank Marianne for her understanding and loving support which gave me a lot of strength during the most stressful final period of this thesis.

The beautiful layout and type setting is due to Sophie Waeber. Her uncomplicated friendly character and the congenial and professional discussions we had made it possible to give this thesis an appearance it deserves.

Last but not least, my parents deserve my deepest gratitude for their constant support, their belief in my abilities and their love.
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Education

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Oral presentations


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13th Congress of the International Society of Ethnobiology. Montpellier, France, Co-presentation with A. Ormsby

“Fragmentation genetics and reproductive success of *Dysoxylum malabaricum* (Meliaceae) in a complex agro-forest landscape”, Ecological Genetics Group 56th Meeting (April 10-12, 2012) Edinburgh, Scotland


“Effects of Fragmentation and Habitat Patch Degradation on the Survival of Native Tree Species in the Sacred Grove Landscape of the Western Ghats, India” (October 25, 2011), Symposium on „Conserving Nature at Sacred Sites: State of knowledge and prospects for research“ Zurich, Switzerland

„Die Kosten des nationalen Biotopschutzes in der Schweiz“ Fachtagung „Was kostet Naturschutz!“ (December 02, 2010), Graz, Austria