UNIVERSIDADE ESTADUAL DE CAMPINAS
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PATRICIA SANAE SUJII

Genetic diversity, structure and mating system of *Centrolobium tomentosum* Guillem ex. Bentham (Fabaceae), to support genetic enrichment in forest restoration areas

Diversidade, estrutura genética e sistema reprodutivo em *Centrolobium tomentosum* Guillem ex. Bentham (Fabaceae) visando subsidiar enriquecimento genético em áreas de restauração florestal

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Thesis presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Genetics and Molecular Biology, in the area of Plant Genetics and Breeding

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Este arquivo digital corresponde à versão final da tese defendida pela aluna Patricia Sanae Sujii, e orientada pela profa. Dra. Maria Imaculada Zucchi

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Os membros da Comissão Examinadora acima assinaram a Ata de defesa, que se encontra no processo de vida acadêmica do aluno.

"Here is the means to end the great extinction spasm. The next century will, I believe, be the era of restoration in ecology"

Edward O. Wilson

(The diversity of life - 1992)

"The most unique feature of Earth is the existence of life, and the most extraordinary feature of life is its diversity"

Cardinale et al. 2012

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Resumo

Diversas iniciativas locais e globais têm sido criadas para reduzir impactos humanos no meio ambiente, reduzindo a perda de habitats com a criação de unidades de conservação, e aumentando a área florestada por meio da restauração florestal. Podemos citar como exemplos dessas iniciativas acordos internacionais como "The New York Declaration on Forests", que tem como meta restaurar 350 milhões de hectares até 2030, e locais, como o Pacto pela Restauração da Mata Atlântica, que visa recuperar 15 milhões de hectares de floresta até 2050. A restauração ecológica requer que, além da cobertura vegetal restabelecida no reflorestamento, também seja recuperada uma comunidade funcional, com populações capazes de sobreviver e se adaptar a mudanças ambientais. Esses grandes investimentos em restauração ressaltam a importância da disponibilidade de informações taxonômicas, ecológicas e genéticas sobre espécies nativas. Entretanto, tanto a composição florística das florestas, como características ecológicas e genéticas de espécies nativas ainda são pouco conhecidas, especialmente em florestas tropicais. Além disso, informações sobre diversidade genética ainda são pouco utilizadas no planejamento de projetos de restauração. Então, este trabalho foi desenvolvido com o intuito de descrever o sistema reprodutivo e a diversidade genética de uma espécie arbórea da Mata Atlântica, comumente utilizada em projetos de restauração florestal, *Centrolobium tomentosum*, e avaliar o sucesso na recuperação da diversidade genética em áreas de restauração. Além disso, desenvolvemos um modelo para compreender os impactos de diferentes níveis de diversidade genética em populações plantadas em áreas de restauração na viabilidade populacional. Nossos resultados indicam que *C. tomentosum* é uma espécie alógama, com limitações na dispersão de semente e grande capacidade de dispersão de pólen. A espécie tem um pequeno potencial de colonização natural de novas áreas, graças à baixa probabilidade de dispersão de sementes a longas distâncias, mas a grande capacidade de dispersão de pólen permite que populações de fragmentos vizinhos sejam funcionalmente conectadas. Ao comparar populações de remanescentes naturais com populações de áreas de restauração, observamos que a restauração é capaz de recuperar diversidade genética, principalmente se as sementes usadas para o plantio forem coletadas de um grande número árvores, reduzindo o efeito fundador. O modelo desenvolvido indicou que a diversidade genética inicial tem efeito sobre a viabilidade populacional, principalmente quando o tamanho da área de restauração e o tamanho populacional inicial são pequenos. As simulações podem ser feitas com espécies com diferentes características e os

resultados podem ser utilizados para subsidiar o planejamento de projetos de restauração ecológica. Os estudos genéticos realizados neste trabalho mostraram-se eficazes para obter um diagnóstico de populações de áreas restauradas sem planejamento quanto à diversidade genética e podem subsidiar o planejamento de novos projetos para a reintrodução de populações em áreas de restauração.

Abstract

Many local and global initiatives have been created to reduce human impacts on environment, reducing habitat loss with delimitation of protected areas, and increasing forest areas with forest restoration. International agreements as The New York Declaration on Forests that has a goal to restore 350 million hectares of forests by 2030, and local initiatives as The Atlantic Forest Restoration Pact that aims to restore 15 million hectares by 2050 are examples of these initiatives. Ecological restoration requires the recovery of both forest cover, which is reestablished in forest restoration, and a functional community, with populations able to survive and to adapt to environmental changes. These large investments in restoration highlights the importance of information about tanoxomy, ecology and genetics of native species. However, neither forest species composition nor ecological and genetics characteristics of native species are well known, especially in tropical forests. In addition, information about genetic diversity is underused in restoration projects planning. Thus, this study was developed to describe the mating system and genetic diversity of an Atlantic Forest tree species widely used for forest restoration, *Centrolobium tomentosum*, and to evaluate the success of ecological restoration to recover genetic diversity. We also developed a model to assess the impacts of different initial levels of genetic diversity on the viability of populations planted in restoration areas. Our results indicated that *C. tomentosum* is an allogamous species, with limited seed dispersal, and large capacity of pollen dispersal. This species has a small potential to colonize new areas, due to the low probability of long distance seed dispersal, but the high potential to long distance pollen dispersal enables functional connectivity of neighbour fragments. Comparing populations from natural remnants to populations from restoration areas, we observed that it is possible to recover genetic diversity with ecological restoration, especially if the seed pool used for the plantation were collected from a large number of seed-trees, reducing the founder effect. The model developed indicated that the initial genetic diversity has a significant effect on population viability, especially in small the restoration area and small initial population size. The simulations can be performed with different species and the results can be used to support planning of ecological restoration projects. Genetic studies presented here have been effective for a diagnosis of populations from areas restored without accounting for genetic diversity and can support planning of new projects for the reintroduction of populations in restoration areas.

Summary

Introduction

Deforestation and forest fragmentation

Over the last 25 years, the rate of net forest loss was reduced in more than 50%, from 0.18% in the 1990s to 0.08% over the last five years. Despite this positive result, the global forested area was reduced in 129 million ha from 1990 to 2015, as human populations grow and more land is necessary for agriculture, pasture and cities development (FAO 2015). The worst situation is observed in tropical regions, more drastically in South America and Africa (Fig. 1).

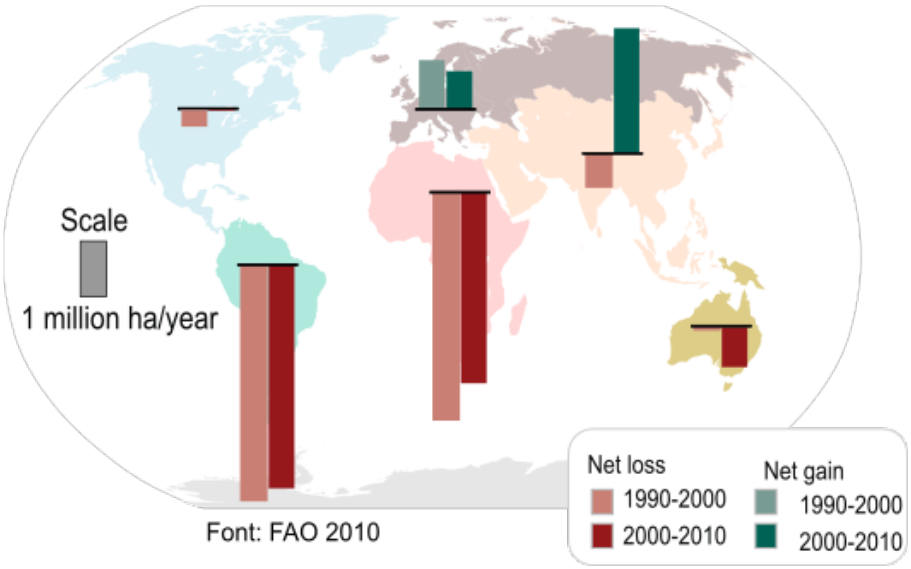

Figure 1. Anual net change in forest area by region. Adapted from FAO 2010.

Both deforestation and forest fragmentation have negative effects on remnant populations. The reduction on forest area leads to decrease in population sizes of most species and fragmentation promotes isolation of remnant populations. The smaller and more isolated is the remnant fragment, stronger are the negative impacts on viability and higher is the probability of local extinction (Matthies *et al*. 2004). In addition to forest cover loss, there is also the loss of environmental services, such as soil and water protection, and support for biodiversity and ecological processes, besides cultural, religious and recreational services (Dobsonet *et al*. 2006).

One way to slow down deforestation or forest conversion is to conserve and protect remnant forests. It is essential for maintenance of biodiversity in many different levels, such as communities, species interactions, species and genes. However, if deforestation and fragmentation levels are

already high, only conservation of remnant fragments may not be enough to preserve viable populations on the long term. One important complement to forest conservation is forest restoration (Huxel & Hastings 1999).

Forest restoration

Forest restoration has an increasing role on the total forest cover worldwide. Since 1990, planted forest areas had an increment of over 110 million ha, and account for 7% of the world's forest area (FAO 2015). These forest plantations comprise both areas restored for biodiversity recovery, production, and other multiple-uses. Ecological restoration, on the other hand, is the "process of assisting the recovery of an ecosystem that is damaged, degraded, or destroyed" (SER 2004). In this context, recovered or restored ecosystems have biotic and abiotic resources to persist and develop without additional human assistance. This requires that the restored area support a functional community, with appropriate species interactions; that the ecosystem is resilient to natural environmental changes and stress; and that there is interaction with surrounding ecosystems in the landscape in terms of both abiotic and biotic flow (SER 2004).

Ecological restoration has many advantages compared to reforestation with monocultures and mixed-species plantings. Restoration plantings are more efficient in carbon biomass storage than monocultures and mixed-species plantations (Kanowski & Catterall 2010). The high species diversity used in ecological restoration projects is associated with resistance to disturbances from pests and pathogens and the self-sustainable environment obtained reduces the dependence on fertilizers and pesticides (Aerts & Honnay 2011).

Forest restoration has been acknowledged as a Clean Development Mechanism of the Kyoto Protocol and, more recently, the United Nations have created mechanisms based on funding and crediting for reducing emissions from deforestation and forest degradation (REDD+). This mechanisms embraces conservation, sustainable management of forests and enhancement of forest carbon stocks in developing countries (Alexander *et al*. 2011). In addition to incentive policies, there are worldwide commitments for forest restoration, such as The New York Declaration on Forests that sets a goal of 350 million hectares restored by 2030, the Green Belt Movement in Kenya that aims to restore millions of hectares of agricultural land (Latawiec *et al*. 2015), and The

Atlantic Forest Restoration Pact that aims to recover 15 million ha of the Brazilian Atlantic Forest by 2050 (Rodrigues *et al*. 2011).

These large-scale projects and investments in forest restoration, especially those that focus on restoration of biodiversity and resilience require taxonomic, ecological and genetic information about native species. For decades, both researchers and restoration practitioners have acknowledged the importance of using mostly native species in restoration projects. More recently, the concern with restoration of ecological processes simulating ecological succession has grown, increasing the focus on the forest ability to self-maintain. On the last decade, there is also the effort to recover populations with high genetic diversity, to enhance resilience, i.e., the potential to recover from environmental changes (Rodrigues *et al*. 2009b; Thomas *et al.* 2014; Suding *et al*. 2015).

Genetic Diversity

Adaptation to changing conditions, such as climate change, arrival of new predators or competitors, and reduction of resources availability, depends on the existence of variability in the population, usually at genetic level (Mills 2013). Populations with high genetic diversity have more raw material for natural selection to act and consequently higher probability to contain individuals fitted to the new condition (Young *et al*. 1996).

This issue is especially important in small and isolated populations, that are more threatened of decline and extinction, because they are more susceptible to effects of genetic drift, i.e., they lose genetic diversity more quickly than larger populations (Ellstrand & Elam 1993; Frankham 2005). In general terms, it is necessary a minimum effective population size (N_e) of 100 to avoid genetic drift effects and inbreeding depression in a short term (5 generations) and N_e of at least 1000 for retaining evolutionary potential for fitness in perpetuity (Frankham *et al*. 2014).

Deforestation and fragmentation usually lead to population reduction, and many studies have shown negative impacts of these bottlenecks on populations genetic diversity and viability (Fahrig 1997; Hobbs & Yates 2003; Honnay & Jacquemyn 2007). Founder effect is a particular case of bottleneck, in which a population is founded from a small sample of a larger population. Thus, in fragments that are result of both natural regeneration process and active restoration, it is possible to observe reduction in genetic diversity due to founder effect. A study of populations in second-growth forests indicated reduced genetic diversity in founding tree populations, reflecting a strong

founder effect, and a shift towards genetically rich populations in the mid- and long-term due to gene flow at the landscape level (Sezen *et al*. 2007). Many of the areas actively restored on the last decades were implanted with high species diversity, but there was little or no concern about genetic diversity (Rodrigues 2009). A study with *Inga vera* tree species in a restoration area also indicated reduced genetic diversity in planted individuals, with higher diversity in seedlings, probably due to gene flow from natural remnants (Neto *et al*. 2014).

On the last decade, however, the importance of genetic diversity for long-term viability of populations in restoration plantations has been highlighted. Both researchers and restoration practitioners have focused on using highly diverse seeds and seedlings to obtain populations with high genetic diversity, and most recommendations are based on the number of seed-trees from which to collect seeds (Bozzano *et al.* 2014). Those recommendations were based on the minimum viable population, and on the mating system of the species. For outcrossing species, it is recommended to collect seeds from at least 30 or 45 plants, depending on the percentage of genetic diversity to be captured (95% or 99%, respectively). For seeds produced by self-pollination, twice as many plants are necessary as seed sources (Sebbenn 2006; Crossa & Vencovsky 2011; Basey *et al.* 2015). The Bureau of Land Management in the United States suggests that seeds should be collected from a minimum of 50 trees, considering that the reproductive biology of most tree species is not well known (USDI BLM 2012).

Consequently, the number of studies that employ a genetic approach within restoration context increased in the last five years. More than half of them (59% of 160 studies) provided information to guide restoration decision-making processes, such as planning translocation of organisms, quantifying demographic changes in target populations, and estimating gene flow over the landscape. However, most tropical regions are poorly studied, as most of these studies were performed in North America, and the implications of restoration on evolutionary processes are also not well understood yet (Mijangos *et al.* 2015). Thus, most decision-making are still based on incomplete knowledge (Rice & Emery 2003; Thomas *et al*. 2014). This fact highlights the need for further case studies especially focusing the genetic diversity of species from neglected and threatened biomes.

Neutral molecular markers are the most commonly used tools for genetic diversity studies in restoration context. They are becoming quicker and more affordable, which makes them more

easily applied in studies that aim to support restoration plans (Mijangos *et al.* 2015). The most commonly used molecular markers are microsatellite markers, because they are affordable, ubiquitous, and have high discriminatory power (Schlötterer 2004). These characteristics allow us to obtain information about genetic diversity, mating system, connectivity, fluctuation on population size, and identifying the origin of individuals (Oliveira *et al*. 2006). This information can be used directly to evaluate the success of restoration projects, and to enhance and support restoration plans, and can be used in simulation models.

Model based studies

In some cases, it is possible to obtain empirical observations of biological processes, and to perform experiments to test different methods and approaches for forest restoration. McIver and Starr (2001) discuss passive and active restoration approaches based on results from scientific literature and indicate when each approach could be more adequate. Bertacchi and collaborators (2015) investigated the effects of understory of restoration sites of different ages on seedling establishment, and observed that the understory of young restoration plantations provides suitable conditions to spontaneous regeneration and enrichment planting of native trees. However, in some cases, experiments can be time consuming and have high costs, which make experimentation unfeasible. The time necessary to execute experiments of long term effects of different initial genetic diversity would make this kind of experiment impracticable, particularly in environments with a large number of species, as tropical forests. In situations as this, models and simulation studies can be a feasible alternative.

Individual-based models (IBM) are simulation models that treat individuals as unique and discrete entities (Grimm 1999). They are especially useful in fields as ecology and landscape genetics, because they enable the incorporation of demographic and environmental stochasticity, that are very common in biological systems. IBM also allows relaxation of assumptions from ideal models, increasing biological realism (Grimm *et al*. 1999). The individual-based simulations are a bottom-up approach, because the focus is first to the individuals (parts) and the way they develop and interact. From the parts, we try to understand properties of the population (system) that emerge from the interaction among these parts (Grimm 1999). This approach is appropriate for hypothesis

testing and for analysis of potential results of field experiments, which can help on optimization of sampling schemes for empirical studies (Epperson *et al.* 2010).

There are some individual-based simulation programs available for population and evolutionary genetics studies. Each program account for a group of parameters that may include population growth, selection, migration, and mutation. They can also account for demographic events such as population size fluctuations, extinction and colonization, among other events (Hoban *et al*. 2012). One example of IBM developed for population viability analysis tested the effects of different managed programs on genetic diversity and demographic viability of populations of orang-utans (Bruford *et al.* 2010). There are fewer studies with plants, and programs rarely accont for the long life spam of trees. One example of a model developed for trees made possible the analysis of the effect of fire, logging and insect attacks on burned area and defoliation of pine trees forests (James *et al.* 2010).

Justification

The Atlantic Forest is one of the hotspots for conservation, with 11.7% remaining from the original extent of primary vegetation (Ribeiro *et al.* 2009). It provides environmental services for over 60% of Brazilian population and is still threatened by deforestation and forest conversion. The Atlantic Forest Restoration Pact was created to restore the Atlantic Forest, conserving biodiversity, while creates employment, income, and helps on legal compliancy of pasture and agricultural activities. The Pact aims to restore 15 million hectares of Atlantic Forest by 2050 (Rodrigues *et al.* 2009a). To accomplish this, more research is necessary to enhance efficiency of restoration practices and to objectively evaluate the restoration success.

Draw populations with high genetic diversity is fundamental to accomplish ecological restoration requirements, as conservation of biodiversity and resilience (Suding *et al*. 2015). Additionally, information about natural levels of genetic diversity and population structure must be available for a large number of species from different successional stages, and with different ecological characteristics (i.e., pollination and seed dispersal, reproductive system, etc.). It is possible to find these information for a limited number of model and threatened species, but most species from tropical forests are still poorly studied (Thomas *et al*. 2014; Mijangos *et al.* 2015). Beside this, the success of restoration projects in recovering genetic diversity is not yet well

understood. Most studies on restoration did not even consider the success of distinct genetic assessments (Ruiz-Jaen & Aide 2005; Wortley *et al*. 2013).

In this study, we aimed to fill this knowledge gap by assessing genetic diversity, population structure, and mating system of a tropical tree species widely used in restoration projects in the Atlantic Forest. The selected species was *Centrolobium tomentosum* Guillem ex. Bentham (Fabaceae).

Centrolobium tomentosum

Centrolobium tomentosum Guill. ex Benth. (Fabaceae) is a tree species widely used in the Atlantic Forest's restoration projects, because it is a typical gap species, with relatively fast growth and symbiotic associations with nitrogen fixation microorganisms (Carvalho 2005; Pagano 2008). This species has a wide range of distribution over the Atlantic Forest and some parts of Cerrado, the Brazilian savanna.

The trees are semi-deciduous, can grow up to 35 m height and can reach 100 cm of diameter at breast height. The trunk is cylindric and straight, the canopy is large and dense (Fig. 2). The species is monoecious, the flowers are observed in inflorescences, with yellow flowers (Siqueira & Oliveira 2000). Flowering period is during the wet season (Aidar 1992; Brina 1998). The main pollinators are large bees with long distance flight capacity (genus Xylocopa, Bombus, Centris, and Megachile) (Aidar 1992), one of the most common group of pollinators of tropical canopy tree species (Bawa 1990).

Fruits are large samaras with approximately 9 g each, and reach maturity at the dry season (Aidar 1992; Brina 1998). Seed dispersal syndrome is anemocory (Cavalho 2005), but most fruits fall under the canopy of the mother tree (Aidar & Joly 2003). Seeds are shade tolerant, but seedling growth is light dependent (Durigan *et al.* 1997).

This species is frequently used for forest restoration, but is also used in urban tree planting, in afforestation of pasture and in agroforestry system (Lorenzi 1998; Toledo Filho & Parente 1988). The tree’s wood can also be explored for construction and other purposes (Carvalho 2005). This species has also phytoterapic uses (Diaz 1992).

Figure 2. *Centrolobium tomentosum* from the restoration area at Cosmopolis municipality (São Paulo State). Left: Adult tree; Right: juvenile.

Study sites

We selected five sample sites for this study. Two of them were restoration areas and three were natural forest remnants. All sites were in included within the semideciduous seasonal forest, one of the most threatened vegetation types of the Atlantic Forest, with only 7% of its natural cover remaining (Ribeiro *et al.* 2009). They were located in São Paulo State, southeastern Brazil, in a region with Cwa climate (Köeppen 1948), surrounded by agricultural and urban areas.

The first restoration area (15 ha) was planted from 1955 to 1960 in the riparian buffers of the Jaguari River in a sugarcane farm in Cosmópolis municipality (São Paulo State). The restoration model used was the random heterogeneous planting, and it was established with high-species diversity (70 species) with predominance of native species (70%), chosen from available seedlings in commercial sources and surrounding landscapes (Nogueira 1977).

The second restoration site (21 ha) was a riparian forest planted from 1988 to 1990, at Iracemápolis municipality (São Paulo State), in the borders of the city's water supply reservoir (Fig. 3) (Brancalion *et al*. 2014). The restoration model was the use of a combination of species from different successional stages in modules of planting (6 pioneers and 2 early secondary, 1 late secondary or climax). This forest patch was also established with high species diversity (140

species), most of them native (77%), chosen from available seedlings in commercial sources (Rodrigues *et al*. 1992).

Figure 3. Restoration area from Iracemápolis municipality (São Paulo State). View from the margin of the water supply reservoir.

The Caetetus Ecological Station (2,170 ha) was a large and well preserved natural forest remnant, surrounded by agricultural areas and pastures (Durigan *et al.* 2000). This population served as a reference of well conserved population for comparisons with the fragmented and restored populations.

The other two areas were natural forest fragments disturbed by historical human-mediated disturbances, such as selective logging and fires. One of them was the Municipal reserve of Santa Genebra Forest, the largest urban semideciduous seasonal forest fragment in São Paulo State (252 ha). It has been compromised by human-mediated disturbances like selective logging, fires, and proliferation of ruderal climbers (Farah *et al*. 2014), thus being chosen as an example of comparison disturbed forest, representing a kind of reference of natural forest submitted to typical chronic disturbances found in the region where the restoration sites are located.

The other disturbed natural remnant (42 ha) was a riparian, second-growth forest surrounded by pasture. It has been through selective logging in the past, but was isolated from the cattle and protected for the last 70 years (Aidar 1992).

Objectives

This study aimed to assess reproductive biology and genetic diversity of *Centrolobium tomentosum*, a tropical tree species frequently used in restoration projects, and support management and restoration plans.

Specific objectives

1. Identify the mating system of a population of *C. tomentosum*;
2. Describe the patterns of seed pollen dispersal of the species in one population;
3. Examine the influence of gene flow by seed and pollen on the spatial genetic structure of populations;
4. Evaluate the success in the recovery of genetic diversity and inbreeding levels in populations from restoration areas, using natural remnants as reference areas;
5. Develop an individual-based model that compares the effects of different initial genetic diversity on population viability;
6. Exemplify the model's applicability.

This thesis is organized in four chapters. Chapter 1 is a description of the nuclear microsatellite markers developed for the species and used for most genetic analyses. Chapter 2 describes aspects of the species reproductive biology. The mating system was identified from the crossing rates, and the patterns of pollen and seed dispersal were estimated from mother-offspring data. The effects of reproductive traits on spatial genetic structure were also discussed in this second chapter. Chapter 3 shows the assessment of the success of restoration projects in restoring genetic diversity of populations. It also contains estimates the variance effective population size in juvenile individuals as a measurement of genetic representativeness. Chapter 4 presents an individual based model that allows us to evaluate the effect of different levels of initial genetic diversity on the population viability in short and mid-terms. Information obtained from this model can be used to support restoration plans, improving genetic diversity recovery in restoration projects. This Thesis ends with a general discussion of its main results and implications for forest restoration.

Chapter 1

Isolation and characterisation of microsatellite markers for *Centrolobium tomentosum* (Fabaceae), a neotropical tree species widely used for Atlantic Rainforest restoration

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Isolation and characterisation of microsatellite markers for *Centrolobium tomentosum* (Fabaceae), a neotropical tree species widely used for Atlantic Rainforest restoration

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Abstract We isolated and characterised eight pairs of primers to amplify microsatellite regions for *Centrolobium tomentosum*, a neotropical tree species widely used for forest restoration, with important pharmacological potential. For the primer characterisation, we genotyped 48 individuals from two populations of *C. tomentosum* from natural remnants of Atlantic Rainforests. We detected 2–9 alleles per locus, observed and expected heterozygosities ranged from 0.08 to 0.72, and 0.08 to 0.83, respectively and we observed private alleles in six of the loci. No linkage disequilibrium was observed and all loci are in Hardy–Weinberg Equilibrium in at least one of the populations. This study presents a powerful tool for population genetic studies of this species.

Keywords Araribá · SSR · Plant genetics · Population genetics

Ecological restoration of forests is a very important complement to forest conservation actions and the knowledge of genetic information can contribute substantially for the development of effective restoration projects and evaluation of restoration success (Thomas et al. 2014). Although molecular technics became more affordable, there are molecular markers available for a small part of neotropical species. *Centrolobium tomentosum* Guillem ex. Bentham (Fabaceae) is a neotropical tree species widely used for forest restoration, with important pharmacological potential (Carvalho 2005). In the present study, we aimed to develop microsatellite primers for *C. tomentosum* and validate them for use in population genetics studies.

We collected samples of *C. tomentosum* from two natural remnants of Atlantic Rainforest in Brazil, both from seasonal semi deciduous forests: Mata de Santa Genebra (22°49′20″S; 47°06′40″W), at Campinas municipality, and Estação Ecológica de Caetetus (22°24′11″S; 49°41′55″W), at Galia municipality, both in São Paulo State. A genomic library was constructed using the protocol developed by Billotte et al. (1999). DNA digestion was performed with the enzyme *Afa I* (Invitrogen). Digested DNA was linked to adapters, amplified by polymerase chain reaction (PCR) and purified using the *QIAquick PCR purification kit* (QIAGEN Cat. # 28106). Fragments with adapters were linked to a complex containing poly-CT/GT oligonucleotide fragments, biotin and magnetic beads (*Dynabeads*—Streptavidin Boehringer Mannhein) and amplified by PCR. These fragments were linked to pGEM-T Easy Vector (Promega) and transformed into *Escherichia coli* strain by chemical transformation. Sequencing reaction templates were generated from 282 transformed clones. Sequencing reactions were performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and purified by precipitation with isopropanol and ethanol. From the 282 DNA fragments sequenced, we designed 79 primer pairs complementary to SSR flanking regions.

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Table 1 Characterization of 15 microsatellite loci for *Centrolobium tomentosum*

Locus	Primer sequence (5′–3′)	Repeat motif	Ta (°C)	Size range (bp)	*A*	Genbank number
Ct 01	F: GGGTGTGGCGATTAGAAAAC	$(TA)_4$ $(CA)_6$ $(CATA)_7$	51	219–249	5	KP284455
	R: TCGAGTTGTAGAAGCGGAATG					
Ct 02	F: TCCAATTATTGTCGGTCTGC	$(CA)_{14}$	51	202–226	5	KP284456
	R: TCAGCAGTGTTAGTATGCCAAG					
Ct 03	F: TGGTGGGAAAGAAGAATACG	$(CA)_7$	51	210–212	2	KP284457
	R: TCTGACTTCAAGGGGGCATA					
Ct 04	F: TGAACAACAAGAGGGGTGAAC	$(ATTTT)_3$	51	155–160	2	KP284458
	R: AATTGCCGTGTCTAGCTTCG					
Ct 05	F: CAACTCAGCAGAGGAACCAC	$(TC)_3$ $(TA)_3$	51	223–229	4	KP284459
	R: CGAGATTCTGTCAACACTTCC	$(TG)_8$ $(TA)_3$				
Ct 06	F: AGCTGAGGTGTGAGGAGTGT	$(AG)_{18}$	55	141–149	7	KP284460
	R: CTGTTCGGGAGCACCTCTA					
Ct 07	F: CGCCGACGTTGAAGATTAGA	$(CA)_5$ TA $(CA)_{10}$ $(TA)_6$	51	178–200	7	KP284461
	R: AGCGAAAGCATGGACAAGAC					
Ct 08	F: GCGAAGAAAATGAAAAGACC	$(CT)_7$ CA $(CT)_8$ $(CA)_7$	55	180–212	9	KP284462
	R: GGAGAGTGTGCCGTAATGT					

Optimal PCR conditions were determined using DNA from two *C. tomentosum* trees from each sampled population. PCR reactions contained 1–5 ng of template DNA, 0.25 µm of each primer, 1U Taq DNA polymerase, 250 µm of each dNTP, 0.25 µg BSA, 1.5 mM $MgCl_2$ and 1 × reaction buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl) in a total volume of 10 µL. Cycling conditions were: 94 °C for 5 min (one cycle), then 94 °C for 1 min, 50 to 56 °C (according to the primer annealing temperature) for 45 s, 72 °C for 45 s (30 cycles); and 72 °C for 7 min (one cycle). We obtained 24 primer pairs which amplified clearly interpretable bands.

To detect loci with intra and inter-population polymorphism we used DNA from six individuals of each population. DNA amplified fragments were separated and analysed using LI-COR 4300 DNA Analyzer (Uniscience). We obtained eight primer pairs that amplified regions with at least two alleles, with clearly identifiable bands (Table 1).

Population analysis was performed with both sampled populations. Linkage disequilibrium was tested and genotypic proportions were tested to Hardy–Weinberg Equilibrium using Fisher's exact test on Genepop on the Web (Raymond and Rousset 1995). Genetic diversity was characterised by estimates of allele numbers, number of alleles per locus, observed heterozigosity and expected heterozygosity under Hardy–Weinberg equilibrium (HWE). Inbreeding coefficient (F_{IS}) was also estimated using Hierfstat (Goudet 2005).

We detected 2–9 alleles per locus and private alleles in seven of the loci. The observed and expected heterozygosities ranged from 0.08 to 0.72, and 0.08 to 0.83, respectively. No linkage disequilibrium was observed and all loci are in Hardy–Weinberg Equilibrium in at least one of the populations. Detailed genetic parameters estimates are presented in supplementary material. This study presents a powerful tool for population genetic studies of this species widely used in forest restoration projects. These are the first microsatellite markers developed for *C. tomentosun*, which are expected to be helpful tools for studies of conservation genetics and reproductive biology of this species widely used in forest restoration projects.

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Chapter 2

High gene flow through pollen partially compensate spatial limited seed dispersal in a Neotropical tree in fragmented and restored forests

Abstract

The negative effects of deforestation and fragmentation can be mitigated by protecting forests remnants, enhancing connectivity among fragments, increasing genetic diversity in remnant populations, and restoring disturbed areas. To accomplish some of these actions, it is necessary to produce seedlings with high genetic diversity. This requires good planing on seed sampling, which is dependent on knowledge on how the diversity is organized in space. This study aimed to support seed sampling plans for conservation of genetic diversity of tropical tree species. To better understand the biology of the species and the structure of genetic diversity in different populations, we tested the following hypotheses: (i) seed dispersal is restricted to short distances; (ii) geographically closer individuals have greater contribution to the pollen cloud; (iii) the species has mixed-mating system; (iv) there is spatial genetic structure in all populations. Using this knowledge, we suggested recommendations for seed sourcing, and seed sampling for conservation and restoration purposes. We estimated seed dispersal distribution indirectly by counting individuals around adult trees, pollen dispersal distribution and outcrossing rates from adult and offspring genotypes, using microsatellite loci. We inferred spatial genetic structure assessing the correlation among kinship coefficients and geographical distances between pairs of individuals, using nuclear and chloroplast microsatellite markers. We observed restricted seed dispersal, with most seeds (78%) falling up to 10 m from the adult tree trunk. The best-fitted pollen dispersal distribution was the exponential power distribution, with a heavy tail and average pollen dispersal distance of 3,191 m. The population analyzed was outcrossing (0.979), with a large number of pollen donors (8.2). We observed significant spatial genetic structure in all populations, with both markers, which suggests that the restricted gene flow by seed dispersal is not completely neutralized by high outcrossing rates and long distance pollen flow. Our results emphasize the importance of conservation and restoration of pollination services in fragmented areas, especially in tropical forests where most species are pollinated by animals and a large number of tree species experience dispersal limitation due to fragmentation and defaunation.

Introduction

Deforestation and forest fragmentation are major threats to species conservation (Dobson et al. 2006), and tropical trees are particularly vulnerable because of demographic and reproductive characteristics. As most tropical trees have high outcrossing rates and suffer from inbreeding depression, the isolation of populations from different fragments and the decline of population size may reduce seed production and increase extinction risk (Cascante et al. 2002; Petit & Hampe 2006; Aguilar et al. 2008; Chaves et al. 2011). Furthermore, the reproduction of many plants is dependent on interactions with pollinators and seed disperses (Bawa 1990; Didham et al. 1996; Ward et al. 2005; Dick et al. 2008), and many studies have shown decline in both vertebrate and invertebrate abundance, and in ecosystem services provided by these animals (Harris & Johnson 2004; Dobson et al. 2006; Dirzo et al. 2014).

The negative effects of deforestation and fragmentation can be mitigated by protecting forests remnants, enhancing connectivity among fragments, and increasing genetic diversity in remnant populations (Lowe et al. 2005; Frankham 2015). Ecological restoration is another effective practice to conserve populations and to reconnect fragments (Dobson et al. 1997; Possingham et al. 2015). In order to increase genetic diversity in remnant fragments and to restore disturbed areas, it is necessary to obtain good quality seeds and seedlings. This includes the production of seedlings with high genetic diversity, which is essential for long-term conservation of populations (Frankham 2005; Basey et al. 2015).

In order to obtain seed with high genetic diversity, it is necessary to understand how the diversity is organized in space. It can be organized in many different ways among populations and over the spatial distribution of individuals in a population. The structure of genetic diversity of plant populations in different levels is influenced by crossing rates and patterns of gene flow by pollen and by seed (Vekemans & Hardy 2004; Hardy & Vekemans 2006; Epperson 2007). Thus information about patterns of seed and pollen dispersal, outcrossing rates and spatial genetic structure are necessary to guide plans of seed sampling for conservation and restoration purposes. Although there are many studies on reproductive biology of tropical tree species (Ward et al. 2005; Azevedo et al. 2007; Collevatti et al. 2008; Fuchs & Hamrick 2011; Tambarussi et al. 2015), and on their seed and pollen dispersal (Bawa 1990; Barbosa & Pizo 2006), the impact of outcrossing and gene flow on genetic structure needs to be better understood (Hamilton 1999; Hardy & Vekemans

2006; Seidler & Plotkin 2006; Sebbenn et al. 2011). Moreover, there is an unfortunate lack of indications of how to use this knowledge in conservation and restoration practices. Some of the few indications include a method to estimate the minimum number of source trees to obtain seed for ex-situ conservation (Sebbenn 2003), and a guide to produce seedlings with high genetic diversity for ecological restoration (Basey et al. 2015).

Objective

This study aimed to support seed sampling plans for conservation of genetic diversity of tropical tree species. We selected *Centrolobium tomentosum* as a model of a Neotropical tree species widely used in forest restoration projects. To better understand the biology of the species and the structure of genetic diversity in different populations, we tested the following hypotheses: (i) seed dispersal is restricted to short distances; (ii) geographically closer individuals have greater contribution to the pollen cloud; (iii) the species has mixed-mating system; (iv) there is spatial genetic structure in all populations. Using this knowledge, we suggested recommendations for seed sourcing, and seed sampling for conservation and restoration purposes.

Methods

Study species

Centrolobium tomentosum Guill. ex Benth. (Fabaceae) is a tree species widely used in the Atlantic Forest's restoration projects, because it is a typical gap species, with relatively fast growth and symbiotic associations with nitrogen fixation microorganisms (Carvalho 2005; Pagano 2008). This species has a wide range of distribution over the Atlantic Forest and some parts of Cerrado, the Brazilian savanna. The distribution of both adults and juvenile individuals is aggregated (Aidar 1992). This is a self-compatible species, and the main pollinators are large bees with long distance flight capacity (genus Xylocopa, Bombus, Centris, and Megachile) (Aidar 1992), one of the most common group of pollinators of tropical canopy tree species (Bawa 1990). Seeds are large samaras (approximately 9 g each), and although the dispersal syndrome is anemocory (Carvalho 2005), most

fruits fall under the canopy of the mother tree (Aidar & Joly 2003), and seeds are hardly dispersed between forest fragments.

Study sites

Study sites were all present within the semideciduous seasonal forest, one of the most threatened vegetation types of the Atlantic Forest global biodiversity hotspot, with only 7% of its natural cover remaining (Ribeiro *et al.* 2009). We collected samples from five populations in São Paulo State, southeastern Brazil, in a region with Cwa climate (Köeppen 1948), surrounded by agricultural and urban areas (Fig.1, Table 1) and embedded in landscapes with very low native vegetation cover (< 10%). The sample sites were classified as: old-growth reference site (Ref), fragmented forest remnant (Frag), and forest restoration (Rest).

Table 1. Description of sample sites of *Centrolobium tomentosum*.

Identification	Fragment classification	area (ha)	Sample sizes	Coordinates
Ref1	Natural preserved	2170	53 juvenile	W 49º42’05" S 22º24'11”
Frag1	Natural disturbed	252	33 juvenile	W 47º06’40” S 22º49'20”
Frag2	Natural disturbed	42	12 adults, 10 juvenile, 8 offsprings (7 - 37 seeds/ offspring)	W 48°10’24” S 22°16’ 03"
Rest1	Restoration	15	47 juvenile	W 47º12’20" S 22º40'18”
Rest2	Restoration	21	64 juvenile	W 47º31’09" S 22º34'36"

The Ref site was Caetetus Ecological Station (2,170 ha), a large and well preserved natural forest remnant, surrounded by agricultural areas and pastures (Durigan *et al.* 2000). The Frag areas were natural forest fragments disturbed by historical human-mediated disturbances, such as selective logging and fires. Frag1 was a disturbed natural forest remnant, the Municipal reserve of Santa Genebra (252 ha), historically compromised by fires, fragmentation, and a harsh matrix

dominated by urbanization and agriculture, which have imposed an arrested succession to the forest (Farah *et al.* 2014). Frag2 was the second disturbed natural forest remnant (42 ha), a riparian, second-growth forest surrounded by pasture. This fragment had selective logging in the past, but was protected since the decade of 1960 (Salis et al. 1994).

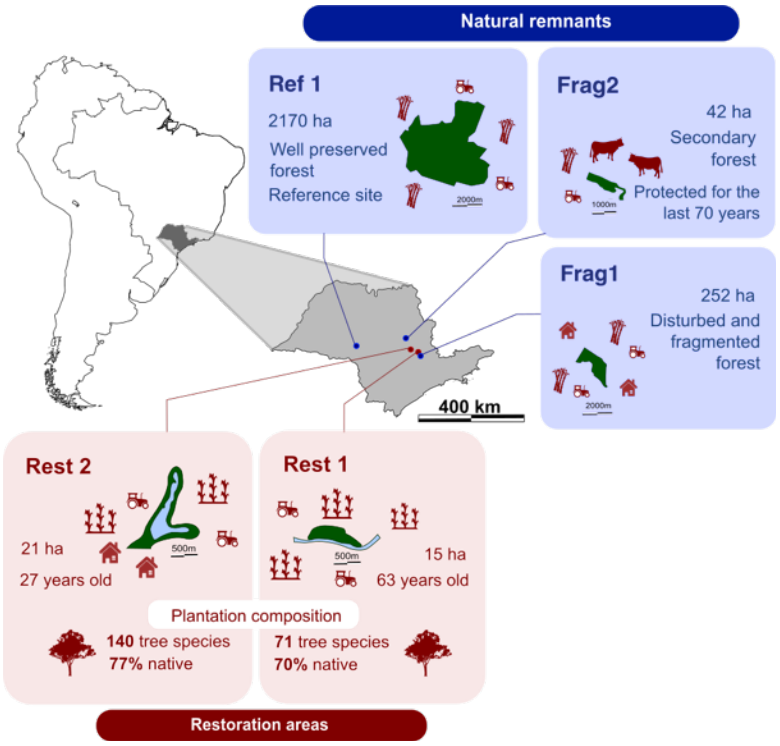

Figure 1. Study sites of *Centrolobium tomentosum* populations, with characteristics of each forest fragment (Ref. = reference site; Frag. = fragmented forest sites; Rest. = forest restoration sites)

The first restoration area (Rest1 – 15 ha) was planted from 1955 to 1960 in the riparian buffers of Jaguari River, in a sugarcane farm in Cosmópolis municipality (São Paulo State). The restoration model used was the random heterogeneous planting, and it was established with high-species diversity (70 species) with predominance of native species (70%), chosen from available seedlings in commercial sources and surrounding landscapes (Nogueira 1977).

Rest2 (21 ha) was a riparian forest planted from 1988 to 1990 on the borders of the city's water supply reservoir in Iracemápolis municipality (São Paulo State). The restoration model was the use of a combination of species from different successional stages in modules of planting (6 pioneers and 2 early secondary, 1 late secondary or climax). This forest patch was also established

with high species diversity (140 species), most of them native (77%), chosen from available seedlings in commercial sources (Rodrigues *et al.* 1992).

Sampling

We sampled plant tissue (leaf or vascular cambium) for DNA extraction from a total of 219 individuals from all sites: spontaneously regenerating juvenile individuals (heigh < 2 m), adult individuals (heigh > 2 m, DAP > 15 cm) (Table 1). We sampled up to 2 juvenile individuals close to each adult tree, to avoid sampling many possibly related individuals. At Ref, we sampled individuals in a small and central portion of the forest area (approximately 10 ha), so the distance between sampled individuals was similar to the other sites. A map with sampling design is presented in Figure 2.

At Frag2, we also selected eight adult trees, and sampled at least 20 open pollinated fruits from each tree (Figure 2). Each fruit of *C. tomentosum* contains from one to three seeds. We germinated the seeds and obtained 137 saplings (7 to 37 saplings/offspring), that were used for mating system analysis.

At Rest 2, we selected 10 adult trees, that were isolated from other *C. tomentosum* trees. We defined five convergent rectangular plots (0.25 x 15 m) around each tree, and each plot was divided in subplots (0.25 x 0.25 m). We counted all juvenile individuals within each of the 60 subplots to infer seed dispersal pattern.

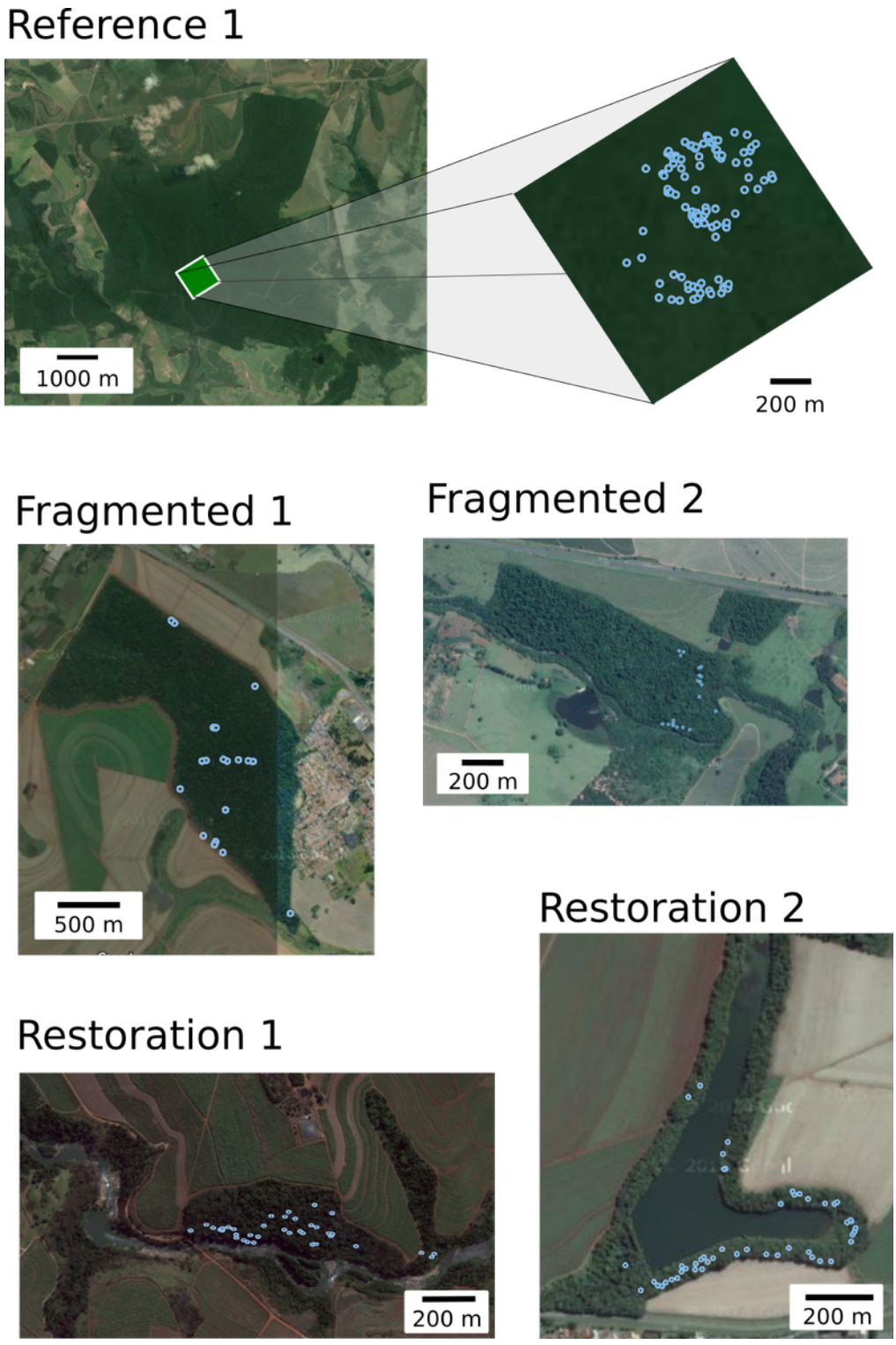

Figure 2. Sampling design of *C. tomentosum* populations in each study area.

Seed dispersal

We analyzed seed dispersal in the Rest2 population. Each of the 15 subplots from a rectangular plot corresponds to a distance class from the trunk of the mother tree. For each distance class, we summed the number of individuals observed in all rectangular plots, and estimated the frequency of individuals of *C. tomentosum*. We used the least square method to fit data of frequency of individuals present in each distance class to different distribution functions using R (R Core Team 2015). The function represents the probability of a seed falling at each distance class apart from the mother tree. We tested one parameter dispersal distributions (normal and exponential), and two-parameter dispersal distributions (exponential power, geometric, and Weibull).

Molecular markers and genotyping

We extracted DNA from all samples using an acid approach for DNA extraction (Cavallari *et al.* 2014). We amplified fragments of DNA from all individuals using seven nuclear microsatellites (nSSR) developed for the species: Ct01, Ct02, Ct03, Ct04, Ct05, Ct07, Ct08 (Sujii *et al.* 2015). DNA samples from Ref1, Frag1, Rest1 and Rest2 were also amplified with five chloroplast microsatellites (cpSSR). The chloroplast microsatellite primers used were: ccmp02, ccmp03, ccmp04, ccmp07, and ccmp10 (Weising & Gardner 1999). Genotypes were obtained using the Li-Cor 4300 DNA Analyzer (Li-Cor Biosciences, Lincoln, NE, USA) and we determined allele lengths using the 50-350bp IRDye700 and 800 (Li-Cor) sizing standard and the Saga v.3.3 software (Li-Cor).

Pollen dispersal

We inferred the contemporary pollen dispersal distance from genotypic data obtained from the mapped adult trees from the site Frag2 and their offspring. We used the software package Poldisp 1.0c (Robledo-Arnuncio *et al.* 2007), that is composed by two modules: KINDIST and TWOGENER. We first tested the correlation of among-sibship correlated paternity and geographic distance, estimating the Pearson correlation coefficient. We then estimated the parameters of the pollen dispersal distribution (scale and shape), the average pollen dispersal distance, and the variance of the pollen dispersal distribution (σ^2) with the KINDIST. We tested one-parameter

(normal and exponential), and two-parameters (exponential power and geometric) dispersal distributions models. We used the results obtained with KINDIST to estimate the global pollen flow structure (Φ_{fi}), and the effective population density (d_e) with TWOGENER.

Mating system

We characterized the mating system for the population from Frag2 and for individual offsprings in this population using the multilocus (t_m) and single locus (t_s) outcrossing estimates. We also estimated the levels of biparental inbreeding (t_m -t_s), proportion of full-sibs among outcrossed sibs (r_p), and the correlation of selfing between two members of an offspring (r_s). We obtained these estimates using the expectation maximization method in the software MLTR (Ritland 2002), following Ritland and Jain (1981) and Ritland (2002).

We estimated the inbreeding coefficient for each seed source tree (F_m) using the SPAGeDi 1.2 program (Hardy & Vekemans 2002). We estimated the fixation index for each offspring (F_o); the total number of alleles (k); the allelic richness (Ar); and the observed heterozygosity (H_O) using diveRsity (Keenan *et al.* 2013) and PopGenKit (Paquette 2012) packages from R (R Core Team 2015). Confidence intervals were obtained with 1,000 bootstrap replicates.

In a partially selfing population, offsprings may be formed as a result of selfing and outcrossing, and a pair of siblings may be both selfed (SS); one selfed and one outcrossed (SO); both crossed with one male parent, i.e. full-sibs (FS); or both crossed with different parents, i.e. half-sibs (HS). We estimated the probabilities of mating events producing: a pair of selfed sibs (P_{SS}), one selfed and one outcrossed pair of sibs (P_{SO}), a pair of half-sibs (P_{HS}), or a pair of full-sibs (P_{FS}) (Ritland 1989);

For this population, we also estimated the effective number of pollen donors (N_{ep} - Austerlitz & Smousse 2001; Smouuse & Sork 2004); the coancestry coefficient (Θ - Ritland 1989), the variance effective population size within offspring (N_e - Cockerham 1969). Using the estimates of crossing rate, inbreeding coefficient for each seed source tree, fixation index for each offspring, effective population size within offspring, proportion of full-sibs among outcrossed sibs, and the correlation of selfing between two members of an offspring, we estimated the number of trees from which sample seed for conservation of genetic diversity (m - Sebbenn 2003), with an effective population size of 100 (Frankham *et al.* 2014).

Spatial Genetic Structure

We analyzed the fine-scale spatial genetic structure (SGS) of all populations. These analyses were performed with nuclear data for all populations and with chloroplast DNA, when available. Chloroplasts DNA is only inherited from the mother-tree for most angiosperm tree species (Corriveau & Coleman 1988), and nuclear DNA is inherited from both parents. Thus, we can assess if there is limitation in seed dispersal, analyzing SGS with cpDNA. We can also evaluate is pollen dispersal is strong enough to neutralize the genetic structure, analyzing SGS with nuclear DNA (Hardy & Vekemans 1999). However, as *C. tomentosum* has an aggregated distribution, and probably SGS has not reached a stationary phase in restoration populations, it is not possible to estimate unbiased pollen and seed dispersal distances SGS data (Vekemans & Hardy 2004).

For the fine-scale spatial genetic structure, we estimated spatial autocorrelation using the J. Nasson's kinship coefficient between pairs of individuals (F_{ij}), as it weights the allele contribution and is not biased by low frequency alleles (Loiselle *et al.* 1995). Average pairwise F_{ij} estimates were plotted against pairwise spatial distances. Distances classes were defined with variable intervals, maximizing the number of pairs of individuals analyzed in each class. For each distance interval, the standard deviation (*SD*) of the average pairwise F_{ij} estimates was obtained using the Jackknife method with 1,000 replications of loci, which was also used to calculate the 95% confidence interval of the pairwise spatial autocorrelation for the null hypothesis of no genetic structure ($F_{ij} = 0$).

The overall extent of spatial genetic structure in each population was quantified by calculating $S_p = b\text{-log}/ (F_1\text{-}1)$, in which *b*-log is the slope of the linear regression between the pairwise kinship and the logarithm of spatial distance between pairs of individuals, and F_1 is the average pairwise kinship between all individuals in the first distance class, which includes all the neighbouring pairs (Vekemans & Hardy 2004). The null hypothesis of absence of structure ($b\text{-log} = 0$) was tested by the Mantel test and significance obtained by 1,000 bootstrap replications. All computations were carried out using the SPAGeDi 1.2 program (Hardy & Vekemans 2002).

Results

Seed dispersal

We obtained the best fit of the distribution of saplings around an adult tree using the Weibull distribution function with scale parameter (λ) = 81.480, and shape parameter (k) = 0.694 (Fig. 2). When $k \leq 1$, the density function is strictly decreasing and the distribution is fat-tailed. Most of the plants counted in the plots (78%) were observed up to 10 m away from the adult tree trunk, but we observed individuals in all other distances from the adult tree in lower frequencies.

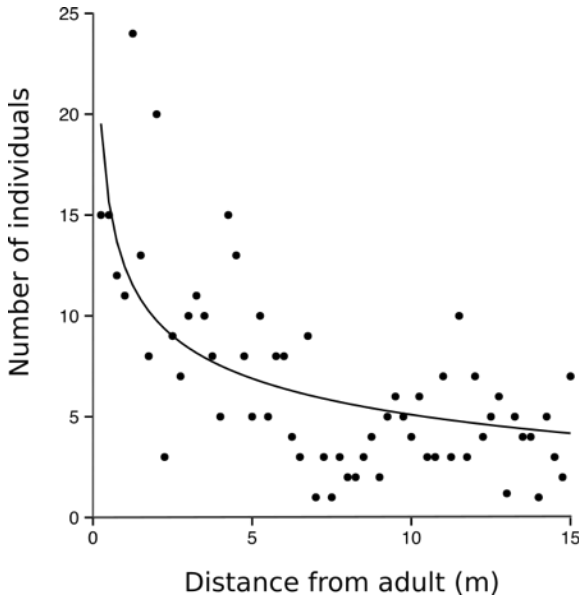

Figure 2. Number of *Centrolobium tomentosum* individuals observed in each distance class from the adult trees. The line represents the Weibull distribution function (λ = 0.6942213, k = 81.4806772).

Pollen dispersal

We observed a significant correlation of among-sibship correlated paternity and separation distance (r = -0.428, p = 0.023). The distribution model with the best fit (smaller least-square residual) was the exponential power distribution (scale = 0.000012 and shape = 0.145) (Table 2, Fig. 3). For this distribution model, the average pollen dispersal distance was 3,191.6 m and variance of the pollen dispersal distribution (σ^2) was 7,519.4. The global pollen flow structure (Φ_{ft}) was 0.085, and the effective population density (d_e) was 0.2 ind/ha.

Table 2. Pollen dispersal estimates for population Frag2 of *Centrolobium tomentosum* population.

Dispersal distribution models	Scale	Shape	Average pollen dispersal distance (δ)	Variance in pollen dispersal distance (σ^2)	Least-square residuals
Normal	13.366	-	11.846	9.45	2.51
Exponential	14.811	-	14.81	12.83	2.45
Exponential power	0.000012	0.14477	3191.61	7519.46	1.80
Geometric	6.247	2.0	Infinite	Infinite	2.14
2D*t*	4.216	1.0	Infinite	Infinite	2.21

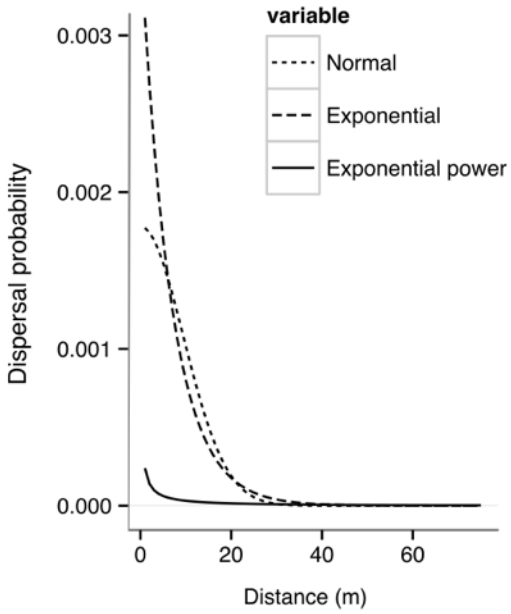

Figure 3. The pollen dispersal kernels estimated for *Centrolobium tomentosum* population Frag2. The best fit was obtained for the exponential power distribution.

Mating system

We observed high outcrossing rates for the population from Frag2 (t_m = 0.979, $CI_{95\%}$ [0.93 - 1.00]), which was consistent for most families (Tables 4 and 5). We also observed evidence of low biparental inbreeding (t_m-t_s = 0.034). The estimated effective number of pollen donors was 8.2, and most of the seeds analyzed were half-sibs (0.84). We observed a small variance effective population

size (N_e = 2.58). The coancestry estimate (Θ = 0.169) was higher than expected under panmixia (Θ = 0.125). Results for the characterization of mating system of the population from Frag2 are presented in Table 2. The analysis of each family resulted in estimates similar to the overall results (Table 5). Our results suggest that if we collect 50 seeds from each tree, we should select at least 36 source trees to conserve an effective population size of 100 (Table 4).

Table 4. Mating system parameters in a *Centrolobium tomentosum* population from a fragmented forest remnant.

Parameter	Estimates
Multilocus outcrossing rate: t_m ($CI_{95\%}$)	0.979 (0.932 - 1.000)
Single-locus outcrossing rate: t_s ($CI_{95\%}$)	0.945 (0.913 - 0.980)
Mating among relatives: t_m - t_s ($CI_{95\%}$)	0.034 (-0.009 - 0.052)
Selfing correlation: r_s (SD)	0 (0.001)
Multilocus paternity correlation: $r_{p(m)}$ ($CI_{95\%}$)	0.122 (0.043 - 0.180)
Effective number of pollen donors: N_{ep} ($CI_{95\%}$)	8.2 (5.56 - 22.73)
Proportion of self-sibs: P_{SS} ($CI_{95\%}$)	0 (0 - 0.004)
Proportion of selfed and outcrossed sibs: P_{SO} ($CI_{95\%}$)	0.04 (0 - 0.125)
Proportion of half-sibs: P_{HS} ($CI_{95\%}$)	0.84 (0.752 - 0.934)
Proportion of full-sibs: P_{FS} ($CI_{95\%}$)	0.07 (0.041 - 0.171)
Coancestry: Θ	0.169
Variance effective size: N_e	2.58
Number of seed-trees: m	39

Table 5. Mating system parameters for each family in a *Centrolobium tomentosum* population.
F_m: inbreeding coefficient for each seed source tree; F_o : progeny fixation index; k: total number of alleles; Ar: allelic richness; H_O: observed heterozygosity; t_m: multilocus outcrossing rate; t_m-t_s: rate of mating among relatives; $r_{p(m)}$: multilocus paternity correlation; N_{ep}: effective

Offspring	Seeds	F_m	F_O (CI$_{95\%}$)	k	Ar	H_O (SD)	H_E (SD)	$\hat{t}_m$ (SD)	$\hat{t}_m$-$\hat{t}_s$ (SD)	$\hat{r}_{p(m)}$ (SD)	N_{ep}	Θ	N_e
M1	18	0.186 (-0.056 - 0.466)	0.128 (-0.583 - 0.008)	21	2.22	0.475 (0.079)	0.051 (0.215)	1.000 (0.000)	0.032 (0.005)	0.117 (0.054)	8.55	0.166	2.66
M2	37	0.393 (0.059 - 0.666)	0.055 (-0.385 - -0.173)	22	2.31	0.52 (0.038)	0.027 (0.160)	0.999 (0.017)	0.026 (0.015)	0.047 (0.022)	21.28	0.183	2.60
M3	7	0.386 (-0.500 - 0741)	0.261 (-0.185 - -0.065)	17	2.27	0.408 (0.101)	0.091 (0.240)	0.993 (0.004)	0.078 (0.018)	0.099 (0.021)	10.10	0.193	2.02
M4	9	0.52 (0.076 - 0.876))	0.13 (-0.301 - 0.113)	20	2.48	0.482 (0.108)	0.08 (0.240)	0.997 (0.002)	0.089 (0.028)	0.070 (0.010)	14.29	0.204	2.05
M5	7	-0.031 (-0.266 - 0.374)	0.029 (-0.543- -0.032)	17	2.30	0.58 (0.126)	0.093 (0.23)	0.982 (0.006)	0.043 (0.008)	0.181 (0.070)	5.52	0.147	2.51
M6	25	0.161 (-0.795 - 0.364)	0.12 (-0.177 - 0.162)	22	2.43	0.447 (0.046)	0.043 (0.212)	1.000 (0.014)	0.110 (0.041)	0.142 (0.065)	7.04	0.166	2.75
M7	7	0.124 (-0.062 - 0.463)	0.065 (-0.267 - 0.514)	15	2.05	0.327 (0.132)	0.110 (0.288)	0.958 (0.066)	0.059 (0.056)	0.109 (0.027)	9.17	0.167	2.28
M8	27	0.294 (-0.120 - 0.612)	0.137 (-0.228 - 0.123)	20	2.10	0.352 (0.058)	0.059 (0.286)	0.768 (0.094)	-0.010 (0.051)	0.055 (0.032)	18.18	0.251	1.90

F_m: inbreeding coefficient for each seed source tree; F_o : progeny fixation index; k: total number of alleles; Ar: allelic richness; H_O: observed heterozygosity; t_m: multilocus outcrossing rate; t_m-t_s: rate of mating among relatives; $r_{p(m)}$: multilocus paternity correlation; N_{ep}: effective number of pollen donors; Θ: coancestry coefficient within progenies; N_e: variance effective size within progenies.

Spatial Genetic Structure

The analysis of spatial autocorrelation with chloroplast DNA indicated that there is significant fine-scale spatial genetic structure in all populations analyzed (Fig. 4 - top; Table 5). The *b-log* values varied from -0.033 to -0.127, all with significance levels smaller than 0.05. For nSSR analysis, the correlation between kinship and geographical distance was significant for all populations, except for Rest1 (Fig. 4 - bottom; Table 6). In all cases, the values obtained for the *Sp*-statistic was higher with chloroplast markers than with nuclear markers (Tables 5 and 6).

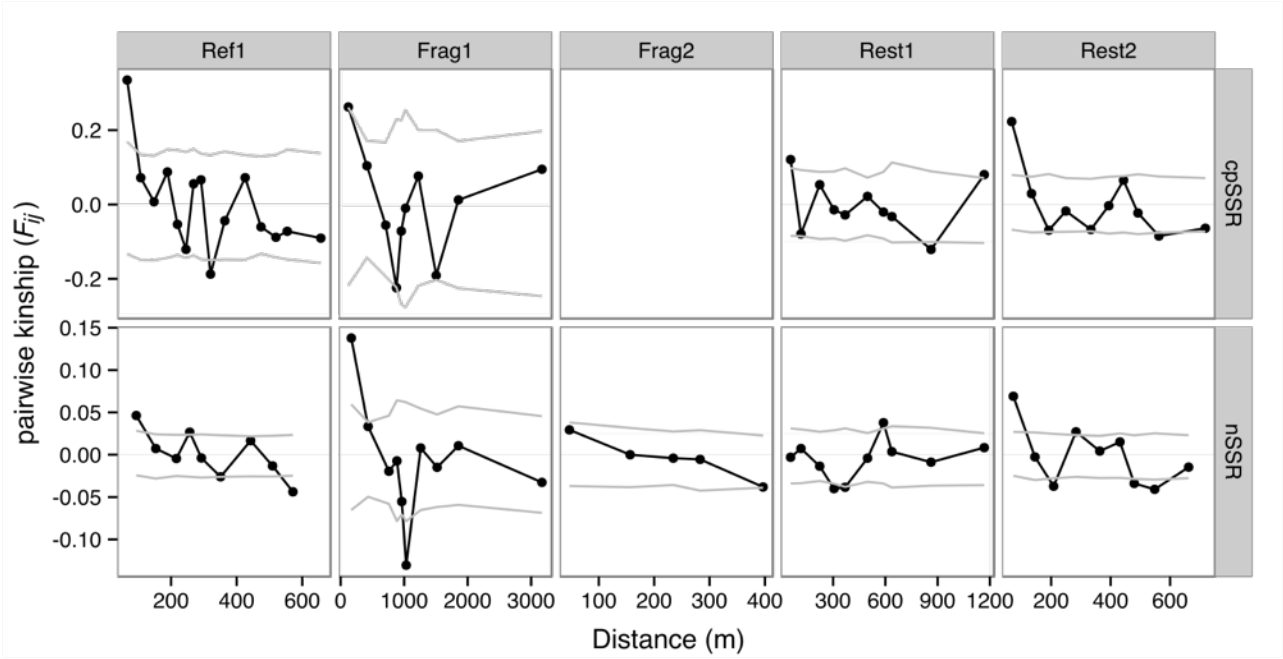

Figure 4. Average pairwise relationship (F_{ij}) over distance intervals for each *Centrolobium tomentosun* population, with chloroplast (top) and with nuclear markers (bottom); gray lines indicate critical values of rejection ($CV_{95\%}$) of the null hypothesis of absence of spatial genetic structure ($F_{ij} = 0$).

Table 6. Estimates of fine scale spatial genetic structure for *Centrolobium tomentosum* populations using chloroplast DNA

cpSSR	F_1	b-log (ln dist)	p-value	Sp statistic	Average N in each distance class	1st distance class
Ref1	0.335	-0.127	0.003	0.191	91.7	66.2
Frag1	0.262	-0.090	0.012	0.122	48.2	120.5
Rest1	0.121	-0.033	0.032	0.038	107	56.1
Rest2	0.223	-0.077	0.000	0.099	142.8	67.2

F_1: average pairwise kinship between all individuals in the first distance class; *b-log*: slope of the linear regression between the pairwise kinship and the logarithm of spatial distance between pairs of individuals; *Sp*-statistic: quantification of spatial genetic structure

Table 7. Estimates of fine scale spatial genetic structure for *Centrolobium tomentosum* populations using nuclear DNA.

nSSR	F_1	b-log (ln dist)	p-value	Sp statistic	Average N in each distance class	1st distance class
Ref1	0.046	-0.023	0	0.024	148.3	94
Frag1	0.138	-0.045	0	0.052	167.5	51.1
Frag2	0.029	-0.022	0.032	0.023	105.4	47.1
Rest1	0.018	-0.004	0.228	0.004	107	56.1
Rest2	0.069	-0.024	0	0.026	159.3	72.7

F_1: average pairwise kinship between all individuals in the first distance class; *b-log*: slope of the linear regression between the pairwise kinship and the logarithm of spatial distance between pairs of individuals; *Sp*-statistic: quantification of spatial genetic structure

Discussion

Seed dispersal is restricted to short distances

The small seed dispersal distance observed in a *C. tomentosum* population is expected due to its type of fruit, large samaras. Although wind dispersed fruits may have long dispersal distances (Seidler & Plotkin 2006), large samaras are usually more poorly dispersed, because of their greater falling speed (Augspurger 1986; Greene & Johnson 1993). This limited potential for long-distance seed dispersal may lead to strong intra-population spatial genetic structure and genetic

differentiation of adjacent populations, which enhances the importance of gene flow through pollen to prevent inbreeding.

In spite of the seed dispersal pattern, with most seeds falling close to the mother tree, we did not observe a large number of individuals in later phases of development (heigh > 2 m) close to the adult tree. This may be due to distance and density-dependent factors, such as attack of pathogens and pests, following Janzen-Connell model (Janzen 1970; Connell 1971). Density dependent attacks of seed predators, herbivores, and pathogenic fungi were observed in studies with different species from tropical forests (Augspurger 1983; Schupp 1992). Higher genetic diversity in the saplings could enhance the probability of persistence of the population, after selective pressures of pathogens, herbivores and predators (Young 1996).

Closer individuals contribute more to the pollen cloud

We observed that geographically closer individuals had greater contribution to the pollen cloud of Frag2 population, but a large amount of pollen was dispersed through long distances. The small value observed for the shape parameter leads to a tail that was heavier than the normal density. This result and the long average pollen dispersal distance are consistent with the long distance capacity of the large size bees that are the main pollinators of this species (Pasquet *et al.* 2008). However, the pollinator behaviour suggests that the estimated average pollen dispersal distance may be overestimated. Large size bees are able to visit hundreds of flowers in a day, but studies showed that the median flying distance is close to 800 m and a large proportion of the flight distances are between 200 m and 1000 m (Pasquet *et al.* 2008; Hagen *et al.* 2011). Researchers compared mean pollen dispersal distance estimated directly using paternity analysis and indirectly from the pollen dispersal curve, and observed that the two-parameter models may overestimate dispersal whereas the one-parameter models may underestimate it (Eduardo *et al.* 2008; Lander *et al.* 2010). Thus, mean pollen dispersal of *C. tomentosum* may be larger than tens of meters and smaller than thousand meters. The pollen dispersal distance estimated for *C. tomentosum* is larger than pollen dispersal distances previously described for species pollinated by wind and smaller animals (Garcia *et al.* 2005; Veron *et al.* 2005; Lander *et al.* 2010; Nielsen & Kjaer 2010; Sebbenn *et al.* 2011), and was similar to pollen dispersal results obtained for species pollinated by large size bees (Silva 2014; Dick *et al.* 2008; Jha & Dick 2010).

We estimated a small effective population density in Frag2 (d_e = 0.2 ind/ha), that was smaller than the actual density of adult individuals in the area (d = 70 ind/ha) observed by Aidar (1992). This indicates that a small proportion of adult individuals contributes to the pollen pool analyzed in this study. This also may indicate that related individuals may be clustered, so the pollen pool that fertilizes flowers from each tree may contain many alleles that are identical by descent. This result indicates that the effective size of this population (N_e) is probably smaller than the census size (N_c), which has important repercussions for conservation. If N_c is larger than N_e, estimates solely of N_c may hide real threats to population long-term viability (Mills 2013).

C. tomentosum is outcrossing

The high outcrossing rate observed for the population from Frag2 indicates that this species is likely outcrossing, as most tree species (Bawa 1990). The predominance of outcrossing may be result of pollination by long-flight animals, the most frequent pollination vector in Neotropical species (Bawa 1990), and as a consequence of longevity and large plant size (Petit & Hampe 2006).

This is a self-compatible species (Aidar 1992), with crossing rates similar to self-incompatible species (t_m=1.0). As we genotyped saplings, instead of seeds, this result may indicate inbreeding depression in early ontogenetic stages (Ayroles *et al.* 2009; Chaves *et al.* 2011). Long-lived perennial species usually have substantial genetic loads, which leads to inbreeding depression, especially in populations with limited seed and pollen dispersal. If there is inbreeding depression in the population, individuals with high homozygosity have a higher probability to die, which leads to higher estimates of outcrossing rates in the offspring (Ward *et al.* 2005).

It would be expected negative effects of fragmentation on plant reproduction, which can be analyzed in terms of patterns of sexual reproduction (Aguilar *et al.* 2006). We observed that t_m estimates in Frag2, a fragmented second-growth forest, was statistically as high as the expected in an allogamous population (t_m = 1.0). This indicates that the patterns of sexual reproduction have a relatively high resilience to fragmentation effects for *C. tomentosum*.

Our results for biparental inbreeding, coancestry and fixation index estimates indicated that there was biparental inbreeding in most offsprings evaluated. Also, the N_e within offspring was smaller than the expected in a panmictic populations (N_e = 4). However, the large number of effective pollen donors indicated that many different trees contributed with pollen for the

production of each offspring. The large number of pollen donors and the long pollen dispersal distance emphasize the importance of conservation and restoration of pollination services in fragmented areas, especially in tropical forests where most tree species are pollinated by animals. This also highlights the importance of sampling seeds from trees surrounded by a large number of trees from the same species (i.e. forest fragments) instead of isolated trees.

We estimated that restoration practitioners should sample seeds from at least 39 trees for conservation of genetic diversity with an effective population size of 100 was 39. This result is in accordance with the recommended 30-50 trees for seed sampling for forest restoration (Sebbenn 2003; Basey *et al.* 2015).

Significant spatial genetic structure

We observed significant correlation between pairwise kinship coefficients and geographical distance obtained with both nSSR and cpSSR analysis for most samples. The SGS observed with cpSSR analysis was probably caused by the limited seed dispersal (Hardy & Vekemans 1999). The significant genetic structure estimated with nSSR analysis showed higher genetic similarities among neighbours than among more distant individuals, as expected under isolation-by-distance. This agrees with the pattern of pollen flow observed in Frag2, the behaviour of the pollinator (Keasar *et al.* 1996; Pasquet *et al.* 2008; Hagen *et al.* 2011) and the aggregated distribution of adults and juvenile individuals of this species (Aidar 1992, Carvalho 2005).

The SGS values (*Sp*-statistic) observed at chloroplast markers were higher than at nuclear markers. This was expected, because the haploid genome present in the chloroplast undergoes the effect of genetic drift twice faster than an outbred diploid genome, as the nuclear. Also, pollen dispersal, which is larger than seed dispersal, does not contribute to gene flow of cytoplasmic DNA.

Our results of SGS for nSSR are similar to the observed in other tree species with limited seed dispersal and pollination by insects. These *Sp*-statistics values were generally larger than values observed in tree species with long distance pollen and seed dispersal (Vekemans & Hardy 2004; Hardy & Vekemans 2006; Dick *et al.* 2008; Ndiade-Bourbou *et al*. 2010). This pattern can be used to group species with similar pollination and seed dispersal syndromes to develop general guidelines for seed sampling.

Implications for conservation

Our analyses of pollen and seed dispersal patterns, outcrossing levels and spatial genetic structure of a tropical tree species provide insights on how these features can be considered in seed sampling for conservation and restoration purposes. As most species with large seeds, with restricted dispersal capacity, *C. tomentosum* has aggregated distribution, and significant spatial genetic structure, so we should avoid sampling seeds from very close trees, that are probably genetically similar. For outcrossing species, with large number of pollen donors, we should prioritize sites with conserved pollination service to be seed sources, to increase probability of sampling seeds with high representativeness of genetic diversity of the population. Finally, our results showed that for an outcrossing species with low to moderate coancestry levels, the number of source trees to collect seeds is close to the minimum number recommended in the literature (30 - 50).

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Chapter 3

Ecological restoration recovers genetic diversity of a Neotropical tree species

Abstract

To support long-term ecological viability of restoration projects, it is necessary to reach adequate levels of genetic diversity in spontaneously recolonizing and reintroduced populations. The importance of genetic diversity in long term viability of populations is acknowledged, but still poorly monitored in restoration projects. This study aimed to monitore genetic diversity and inbreeding levels of populations of a tree species widely used in restoration projects in the Atlantic Forest, *Centrolobium tomentosum*, exploring the potential of active restoration to successfully reestablish populations with higher chances of long-term perpetuation in agricultural landscapes. We used both nuclear and chloroplast microsatellite markers to assess genetic parameters in juveniles and adult individuals in two restoration areas (28 and 60 years old), one disturbed fragment, and one large and well conserved protected area, located within the Atlantic Forest in SE Brazil. We observed similar levels of genetic diversity and inbreeding in both restored and natural populations, for juveniles and adults. Surprisingly, haplotype diversity was higher in restoration sites. We also found private alleles in juveniles in both restoration areas, which are evidences of gene flow between restored and neighbouring natural populations. However, we observed negative effects of inbreeding on the effective population size of populations from the disturbed natural remnant and restoration areas. These results provide evidences of the capacity of recovery of levels of genetic diversity in restoration plantations and of the importance of maintaining large and well conserved forest remnants to be used as seed sources for restoration efforts.

Key-words

population genetics, *Centrolobium tomenstosum*, Atlantic Forest, inbreeding, effective population size

Introduction

Many international initiatives of ecological restoration have been launched to mitigate negative consequences of deforestation, habitat fragmentation, and other anthropogenic impacts on biodiversity and human wellbeing (Chazdon *et al*. 2016). These projects aim to restore millions of hectares of forest ecosystems and landscapes on the next decades and reestablish new populations of native tree species where they were locally extinct (Latawiec *et al.* 2015). The massive financial investments and political commitments to support restoration programmes highlight the importance of enhancing the process efficiency and the development of reliable monitoring approaches to safeguard key ecological principles for sustaining restoration success (Suding *et al*. 2015).

Biodiversity monitoring in restoration projects has been mostly focused in taxonomic diversity (Ruiz-Jaen & Aide 2005; Wortley *et al*. 2013) and functional diversity (Holl & Brancalion 2016), with few studies on phylogenetic (Schweizer et al. 2015) or genetic diversity (Rodrigues 2013; Neto et al. 2014). Consequently, little is known about the potential of restoration interventions to reestablish similar genetic diversity levels in relation to reference ecosystems. Although some conceptual frameworks have been recently proposed to monitore genetic issues in restoration projects (Thomas *et al*. 2014; Mijangos *et al*. 2015), on-the-ground assessments are scarce (Salas-Leiva *et al*. 2009; Neto *et al.* 2014).

Inbreeding levels is another genetic parameter with importance for monitoring reintroduced populations in restoration programs, since the mating of closely related individuals may lead to a reduction in fitness-related traits such as survival or fertility, a phenomenon called inbreeding depression (Charlesworth & Willis 2009). In plants, inbreeding depression is more common in perennial trees than in annual herbs (Angeloni *et al*. 2011), thus inbreeding in long-lived trees could reduce the chances of population viability in restoration areas. Therefore, it would be reason of concern if inbreeding coefficients in tree populations of restoration areas were higher than their normal levels found in natural, conserved populations, as observed in populations of *Avicennia germinans* (Salas-Leiva *et al*. 2009), and *Inga vera* (Neto *et al.* 2014).

The use of genetics as a source of information to support decision making in restoration programs is particularly relevant in developing tropical countries, where most of the global biodiversity hotspots are located (Myers *et al*. 2000). However, few restoration genetics studies have been carried out there, and the concern about genetic diversity levels in restoration sites started

just a few decades ago (Thomas *et al*. 2014; Mijangos *et al*. 2015). Genetic studies on tropical trees in restoration projects may provide a necessary knowledge platform to plan, implement, and monitor the ambitious restoration programmes planned for tropical regions, including the ambitions goals to bring to 20 million hectares of forest ecosystem and landscapes to restoration in Latin America and Caribbean by 2020 (WRI 2016), to restore 15 million hectares of the Atlantic Forest biodiversity in Brazil hotspot by 2050 (Melo *et al*. 2013), and many other commitments established internationally by the Bonn Challenge, the United Nations Climate Summit, and the Aichi target 15 of the Convention on Biological Diversity (Suding *et al*. 2015).

In order to shed light onto the potential of forest restoration to recover genetic diversity and inbreeding to levels observed in natural remnants, we tested the following hypotheses: (i) restoration areas were implanted in the past with low genetic diversity; (ii) populations from restoration areas have lower levels of genetic diversity than observed in natural remnants; (iii) populations from restoration areas have higher levels of inbreeding than those from natural remnants; (iv) there is gene flow between restoration areas and neighboring areas.

Material and Methods

Study species

We selected *Centrolobium tomentosum* Guill. ex Benth (Fabaceae) as model for this study, because this is a species widely used in the Atlantic Forest's restoration, and is self-compatible (Aidar 1992), which may lead to high levels of inbreeding in the absence of pollinator. Therefore, this is a suitable model for assessing the limitation and the potential of restoration to recover adequate levels of genetic diversity and gene flow to sustain population persistence in restoration sites.

C. tomentosum is a typical gap, intermediate succession species, with relatively fast growth and symbiotic associations with nitrogen-fixing microorganisms (Carvalho 2005; Pagano 2008). The main pollinators are large bees with long distance flight capacity (genus *Xylocopa, Bombus, Centris,* and *Megachile*) (Aidar 1992), which is one of the most common group of pollinators of tropical canopy tree species (Bawa 1990). Fruits are large samaras (approximately 9 g each) dispersed by wind, but most fruits fall under the canopy of the mother tree (Aidar & Joly 2003).

Although this species is self-compatible, most seeds that germinate are result of outcrossing (Sujii et al. unpublished data).

Study sites and sampling

We selected four sample sites, all located within the seasonal semideciduous forest domain of the Brazilian Atlantic Forest of São Paulo state, southeastern Brazil, a global hotspot for biodiversity conservation (Myers *et al.* 2000). This is one of the most threatened vegetation types of the Atlantic Forest, with only 7.5% of its natural cover remaining (Ribeiro *et al.* 2009). All sites had a Cwa Köppen climate and were embedded in human-modified landscapes, dominated by sugarcane plantations, pastures, or urban areas.

We evaluated populations from two restoration sites. The first restoration area (Rest1) was implanted between 1955 and 1960, in a sugarcane farm in Cosmópolis municipality, a 15 ha restoration plantation established along the riparian buffer of the Jaguari River. The restoration model used was the random distribution of a high diversity of trees (71 species; 70% native), regardless of their successional performance, in density of 833 individuals per hectare (Nogueira 1977; Schweizer et al. 2015). The second restoration area (Rest2) was also implanted in a sugarcane farm, from 1988 to 1990, in Iracemápolis municipality. This fragment has 21 ha, and it is also a riparian forest, but planted surrounding the city's water supply reservoir (Brancalion *et al*. 2014). The restoration model was the use of a combination of species from different successional stages in modules of planting (6 pioneers and 2 early secondary, 1 late secondary or climax). This forest patch was also established with high species diversity (141 species), most of them native (77%), chosen from available seedlings in commercial sources (Rodrigues *et al*. 1992).

We selected two natural remnant areas to compare with the forest restoration sites. The first natural remnant (Ref) was the Caetetus Ecological Station, the largest (2170 ha) and best preserved forest patch of the region, surrounded by agricultural areas and pastures (Durigan *et al*. 2000), chosen as the reference ecosystem for this study. The second natural remnant (Frag) was the Municipal reserve of Santa Genebra Forest, the largest urban semideciduous seasonal forest fragment in São Paulo State (252 ha). It has been compromised by human-mediated disturbances like selective logging, fires, and proliferation of ruderal climbers (Farah *et al*. 2014), thus

representing a typical forest remnant of the regions, exposed to chronic human-mediated disturbances and strong edge effects.

We sampled a total of 343 adult and juvenile individuals. From each restoration area, we sampled all adult individuals and more than 30 juveniles. From each natural remnant, we sampled at least 30 adults and 30 juveniles (Table 1). Up to two juvenile individuals were sampled close to each adult individual, to avoid sampling a large proportion of siblings. From each individual, we collected plant tissue (leaf or vascular cambium) for DNA extraction, and obtained the coordinates with a GPS (GPSMAP62, Garmin).

Table 1. Description of study areas and sample sizes.

Study area	Fragment group	area (ha)	Sample sizes	Coordinates	Other information
Ref	Natural preserved	2,170	46 adult 50 juvenile	W 49º42’05" S 22º24'11”	Preserved since the beginning of farming in the region and protected as an Ecological Station since 1987 (SMA 1998)
Frag	Natural disturbed	252	32 adult 33 juvenile	W 47º06’40” S 22º49'20”	Preserved since the beginning of farming in the region and protected since 1981, with recent drastic changes in community structure (Farah *et al.* 2014)
Rest1	Restoration	15	19 adult 47 juvenile	W 47º12’20" S 22º40'18”	Restored with 71 tree species, 70% native (Nogueira 1977)
Rest2	Restoration	21	52 adult 64 juvenile	W 47º31’09" S 22º34'36"	Restored with 141 tree species, 77% native (Rodrigues *et al.* 1992)

Genotyping

We extracted DNA from all samples using an acid approach for DNA extraction (Cavallari *et al.* 2014). We amplified fragments of DNA from all individuals using seven nuclear microsatellites (nSSR) developed for the species: Ct01, Ct02, Ct03, Ct04, Ct05, Ct07, Ct08 (Sujii *et al.* 2015). We also used five universal chloroplast microsatellites (cpSSR): ccmp02, ccmp03, ccmp04, ccmp07, and ccmp10 (Weising & Gardner 1999). The nuclear loci analyzed were in

linkage equilibrium and were considered as independent loci. The chloroplast genotypes were organized in haplotypes.

Genotypes were obtained using the Li-Cor 4300 DNA Analyzer (Li-Cor Biosciences, Lincoln, NE, USA) and we determined allele lengths using the 50-350bp IRDye700 and 800 (Li-Cor) sizing standard and the Saga v.3.3 software (Li-Cor).

Genetic analyses

To analyze genetic diversity of maternal lineages, we organized chloroplast genotypes in haplotypes and estimated haplotype diversity using the Shannon's index (Brown & Weir 1983). The number of haplotypes was used to assess the genetic diversity in the seed pool used for the forest restoration. The number of haplotypes observed in each sample should be equal to or smaller than the number of trees used as seed source, because it is possible that more than one source tree shared the same haplotype. The haplotype number and frequency were estimated with the software Arlequin 3.1 (Excoffier *et al*. 2006). Shannon's index values were estimated using the package Vegan (Oksanen *et al*. 2015) in R (R Core Team 2015).

To estimate the nuclear genetic diversity for adults and juveniles separately and for all individuals in the population, we used expected heterozygosity under Hardy-Weinberg Equilibrium (H_E), observed heterozygosity (H_O), and allelic richness (Ar). We also compared Wright's fixation index (f) in different life stages and populations, estimated from nuclear genotypes for adults, juveniles, and populations. These parameters were estimated using diveRsity (Keenan *et al*. 2013) and PopGenKit (Paquette 2012) packages from R (R Core Team 2015). Confidence intervals were obtained with 1,000 bootstrap replicates.

We estimated the variance effective population size (N_e) of adult and juvenile individuals based on Cockerham (1969), accounting for the sample size (N), coancestry (Θ) and inbreeding, infered from the fixation index (f). The coancestry was inferred from the kinship coefficient using the estimator of J. Nasson (Loiselle *et al.* 1995), using the SPAGeDi 1.2 program (Hardy & Vekemans 2002). The confidence intervals were obtained with 1,000 bootstrap replicates, resampling loci. We also estimated the genetic representativeness (N_e/N) of each sample.

We assessed the number and frequency of private alleles in nuclear genotypes (pA) and the private allelic richness (pAr) in different life stages to look for indications of gene flow, using the

ADZE software (Szpieh *et al*. 2008). Private allelic richness provides a measure of the singularity of each sample (Rodrigáñez *et al*. 2008).

Results

The haplotype diversity in restoration areas was higher than in natural remnant fragments (Fig. 1). In natural remnant populations, we observed three (Ref) or four (Frag) haplotypes both in adult and juvenile samples. In populations from restoration areas, we observed six haplotypes for Rest1 and 13 haplotypes for Rest2. The Shannon's index was higher in samples from restoration areas than in samples from natural remnants (Fig. 1). Only one haplotype was observed only in juvenile samples (H11). The number of haplotypes observed in juvenile samples was always equal or smaller than the observed in adult samples, in spite of the larger sample size of juveniles.

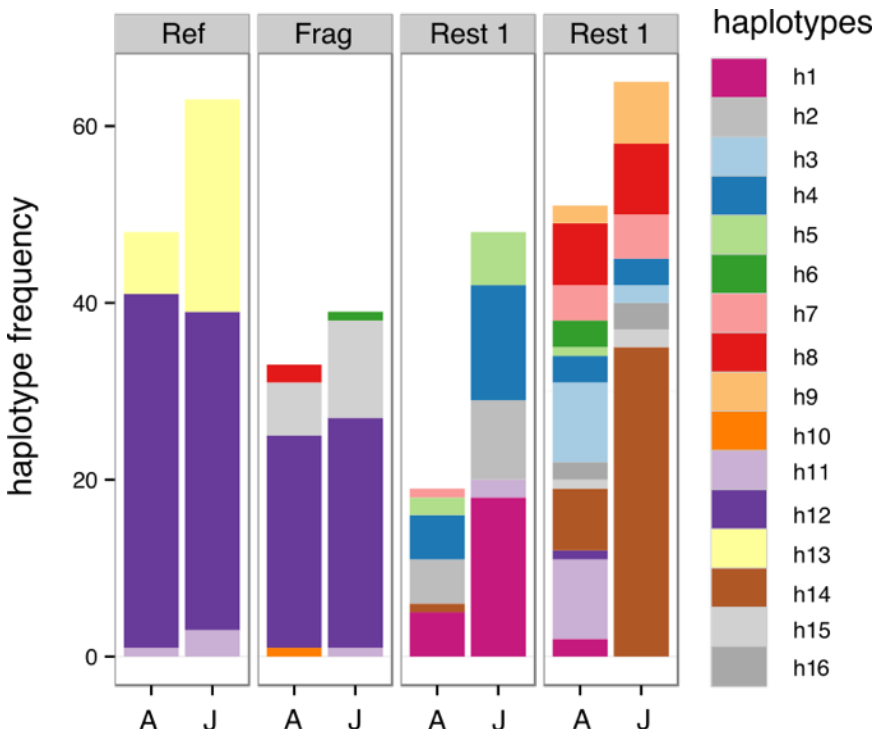

Figure 1. Haplotype diversity in *Centrolobium tomentosum* populations from natural fragmented (Frag) and conserved remnants (Ref) and restoration areas (Rest1, Rest2) for adult (A) and juvenile (J) samples. Bars represent absolute frequency of each haplotype and he numbers above bars are Shannon's index.

The estimates of genetic diversity (H_O and *Ar*) were similar in all samples from all populations (Fig. 2). The estimates of expected heterozygosis (H_E) for both adults and juveniles

from Rest1 were lower than for the other populations. Inbreeding levels were similar among populations (Fig. 2). Comparing juvenile samples, only inbreeding level for Ref population was not consistently different from zero (f = 0.040, $CI_{95\%}$ [-0.046 - 0.105]).

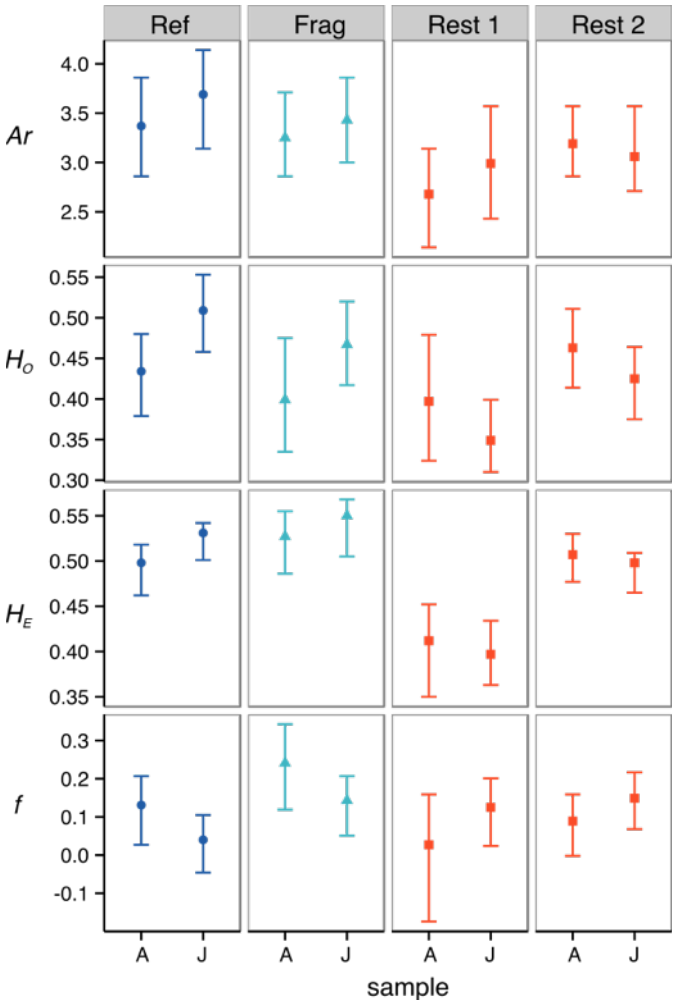

Figure 2. Estimates of genetic diversity and inbreeding levels in *C. tomentosum* populations from natural remnants (Ref, Frag) and restoration areas (Rest1, Rest2) for adult (A) and juvenile individuals (J). *Ar*: allelic richness; H_O: observed heterozygosity; and H_E: expected heterozygosity under Hardy-Weinberg equilibrium; *f*: inbreeding coefficient. Bars indicate 95% confidence intervals.

We observed private alleles from juvenile samples in both restoration areas. In Rest1, we observed nine private alleles (19%) and in Rest2 we observed three (5%). As we sampled all adult individuals in these areas, this result indicated that new alleles were introduced in the restoration area by gene flow from surrounding areas. The private allelic richness was higher in juvenile samples from restoration areas, when compared to adult samples (Table. 2).

Table 2. Private allele (*pA*) and private allelic richness (*pAr*) in *Centrolobium tomentosum* populations from restoration areas.

Population	*pA* in juveniles	*pAr* (Standard Error)	
		Adults	Juveniles
Rest1	9 (19%)	0.014 (0.016)	0.224 (0.136)
Rest2	3 (5%)	0.004 (0.003)	0.135 (0.094)

The coancestry estimates were all very close to zero, indicating that samples were not related (Table 3). The effective population size (N_e) of adults and juveniles from the Ref area were not significantly different from the sample size (N). All juvenile sample sizes from the Frag and Rest areas were significantly larger than N_e (Fig. 3). The genetic representativeness was higher than 80% for all samples (Table 3).

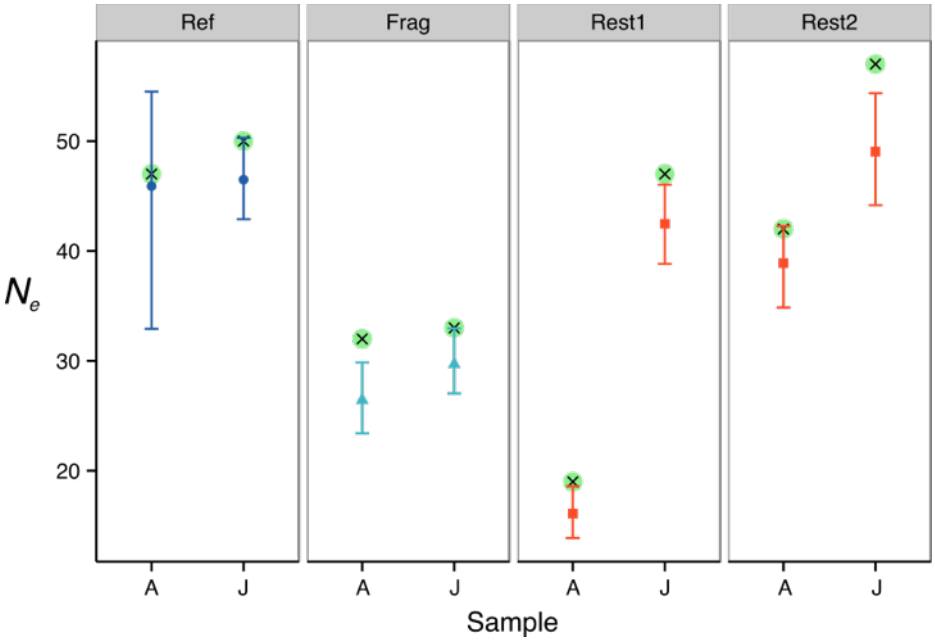

Figure 3. Variance effective population size (points, triangles and squares) of adult (A) and juvenile (J) samples of *Centrolobium tomentosum* from natural remnants (Ref, Frag) and restoration areas (Rest1, Rest2). Error bars represent 95% confidence intervals; and circles with crosses represent sample sizes.

Table 3. Estimates of fixation index (*f*) with the confidence interval ($CI_{95\%}$), coancestry (Θ), effective population size (N_e), and genetic representativeness of each sample (N_e/n), and the sample size (n).

Sample	n	*f* ($CI_{95\%}$)	Θ	N_e ($CI_{95\%}$)	N_e/n
Ref - Adults	47	0.131 (0.027-0.207)	0	45.90 (54.74-32.27)	0.98
Ref - Juvenile	50	0.040 (-0.046-0.105)	0	46.48 (50.30-43.04)	0.93
Frag - Adults	32	0.241 (0.119-0.343)	0	26.42 (29.79-23.41)	0.83
Frag - Juvenile	33	0.143 (0.051-0.207)	0.00059	29.70 (32.54-27.12)	0.90
Rest1 - Adults	19	0.027 (-0.174-0.159)	0.00327	16.11 (18.71-13.84)	0.85
Rest1 - Juvenile	47	0.125 (0.024-0.201)	0.00014	42.47 (46.29-38.74)	0.90
Rest2 - Adults	42	0.089 (-0.002-0.159)	0.00062	38.90 (42.47-34.40)	0.93
Rest2 - Juvenile	57	0.149 (0.068-0.217)	0.00006	49.04 (54.63-44.11)	0.86

Discussion

Overall, *C. tomentosum* populations in restoration sites had comparable levels of genetic diversity, coancestry and inbreeding to those in natural forest remnants, as well as higher haplotype diversity. These results evidence that both restoration areas were implanted with seedlings of high genetic diversity, probably from different sources.

Restoration areas were implanted in the past with high genetic diversity

Our results did not show evidences of founder effect in populations from restoration areas. The chloroplast DNA analysis of adult individuals showed the presence of six different haplotypes in Rest 1, which indicates that seeds were sampled from at least six mother trees. In Rest2, the number of seed sources was even higher (n=13). The haplotypes diversity observed in restoration areas was higher than the diversity in populations from remnant forests, suggesting that the seeds used in the restoration project were sampled from many seed-trees, probably from different forest

fragments. The analysis of genetic diversity of nuclear DNA from adult samples did not show reduced allelic richness and heterozygosity, which are signs of founder effect (Hartl & Clark 2010).

Such positive strategy for genetic conservation is aligned with the long history of scientific and practical maturation of ecological restoration in the Atlantic Forest, which now promotes the use of high levels of both species and genetic diversity in restoration plantations (Rodrigues *et al*. 2009; Brancalion *et al*. 2012). An additional factor that may have contributed to the high level of genetic and haplotype diversity found in this work is the big size of *C. tomentosum* seeds, because many mother trees have to be harvested in order to obtain a given amount of seeds compared to species bearing small- to medium-sized seeds.

Analyzing samples from restoration areas, we observed lower Shannon's diversity index in haplotypes of juvenile individuals than in adults. However, this pattern was not observed in natural remnant populations. This may be a result of genetic drift effect, due to the small population size in restoration areas (Ellstrand & Elam 1993). This may also be a consequence of outbreeding depression caused by interaction of nuclear and cytoplasmic genomes (Scopece et al. 2010). If seeds used in the restoration project had very different provenances, mating among individuals from different origins may reduce seed or sampling viability (Scopece *et al.* 2010; Pinheiro *et al.* 2013). Thus, high levels of genetic diversity, especially when it is obtained from very distant populations, is not always beneficial to restoration success.

Populations from restoration areas have similar levels of genetic diversity to observed in natural remnants

The evaluation of genetic diversity in this study was based on allelic richness, observed proportion of heterozygote genotypes and allele frequencies of neutral regions of the genome. These estimates indicated that genetic diversity was similar in populations of restoration areas and natural remnant populations. Neutral genetic diversity has long been studied both in well preserved and disturbed, fragmented populations in order to assess the conservation status of targeted species (Lowe *et al.* 2005; Honnay & Jacquemyn 2007; Aguilar *et al*. 2008; Pautasso 2009; Vranckx *et al*. 2012; Lowe *et al*. 2015). Although high genetic diversity is not a guarantee of potential to adaptation, it has already been detected a significant correlation between neutral levels of genetic

diversity and population fitness (Reed & Frankham 2003), thus allowing inferences on the potential of population persistence in restoration sites in relation to its levels of genetic diversity.

The results of genetic diversity for juvenile samples indicated that there was no evidence of negative effects in genetic diversity in the first few generations after implantation. Studies with *Myroxylon peruiferum*, *Piptadenia gonoacantha*, and *Casearia sylvestris*, tree species from the Atlantic Forest, in the same areas of our study also showed that populations in restoration areas can have genetic diversity as high as populations from natural remnants (Zucchi *et al*. unpublished data). Studies with *Hymenaea stigonocarpa* and *Dipteryx alata*, tree species from Cerrado (Brazilian Savanna), showed larger number of alleles in restoration areas, when compared to natural remnants, probably due to mixture of seeds from different forest fragments to produce seedlings to be used in the restoration project (Rodrigues 2013). Thus, although previous research has shown that the fragmentation of continuous forest patches into many small patches reduces genetic diversity of trees (Honnay & Jacquemyn 2007; Aguilar *et al*. 2008; Lowe *et al*. 2015), the re-creation of small patches of forest through ecological restoration may re-establish similar or even higher levels of genetic diversity compared to both fragmented and conserved forest remnants. This positive result relies on the key role of seed collection for improving planting stocks in forest restoration, as already demonstrated for the same region of study (Brancalion *et al*. 2012).

Populations from restoration areas have similar levels of inbreeding to those from natural remnants

Inbreeding levels of populations from restoration areas were not significantly different from natural remnant populations. However, we observed that for juvenile individuals, the only sample with inbreeding coefficient not significantly different from zero was the well preserved natural remnant. Also, we observed high estimates of inbreeding levels in the disturbed natural fragment. As *C. tomentosum* is an outcrossing, the inbreeding may be an indication of mating among relatives caused by deficit of pollination services in restoration areas (Rest1 and Rest2) and disturbed fragment (Frag). Although the reestablishment of pollination services in restoration areas is now well understood, it is known that specialized plant-pollinator interactions are more difficult to be successfully recovered, and that highly fragmented landscapes may not adequately support pollinator migration to restored sites (Dixon 2009). Thus indications of pollination service deficits in restoration areas are matter of concern and should be more thoroughly investigated.

As expected, the N_e and the sample size were similar in both samples from the well preserved natural remnant, indicating that inbreeding and coancestry do not have negative effect on genetic diversity in the studied populations. For most samples from fragmented and restoration areas, the N_e was significantly smaller than the sample size. This is an effect of inbreeding or crossing among relatives, which may be due to the small population size, associated to spatial genetic structure and deficit of pollination service (Sujii et al. unpublished data). Although, the genetic representativeness was high for all samples, which indicates maintenance of genetic diversity over the next generations (Vencovsky & Crossa 2003; Raposo *et al*. 2007), in both restoration areas, where all adult individuals were sampled, the effective population sizes were smaller than the recommended for short-term (Ne ≥ 100) and long-term (Ne ≥ 1000) conservation of populations (Frankham 2014). This emphasizes the importance of enhance connectivity among surrounding fragments.

There is gene flow between restoration areas and neighboring areas

In restoration areas, we sampled all adult individuals, so if an haplotype is present exclusively in juveniles, it can indicate that the seeds dispersed from trees in neighbour areas or that the parental tree was already dead. Only one haplotype (H11) was present in juveniles and absent in adults in a restoration area (Rest 1), and it was observed in only two juvenile individuals. This absence of evidences of gene flow by seed dispersal was expected for a species with a large fruit dispersed by wind, with low seed dispersal capacity (Greene & Johnson 1993; Sujii et al. unpublished data).

The presence of private alleles in juvenile samples of restoration areas is an evidence of gene flow from neighbour areas. The *pAr* estimates for juvenile samples from restoration areas were significantly higher than for adult samples. As we sampled all adult individuals from the restoration areas, private alleles in juveniles probably came from neighbour areas, most likely by pollen flow. This evidence of gene flow between restoration areas and the surrounding natural remnants indicates that restoration populations can be allele source for previously isolated fragments, contributing to the increase of the effective population size in the set of neighbour populations. This is a yet poorly studied contribution of restoration to biodiversity conservation in human-modified landscapes. However, it may also be a source of a problem for native populations' conservation,

since these new alleles may cause outbreeding depression, *i.e.*, decline of progeny fitness by the crossing of individuals adapted to different conditions (Lesica & Allendorf 1999).

Implications for practice

Our results corroborate other studies that showed evidences of ecological restoration capacity to recover genetic diversity (Smulders *et al*. 2008; Ritchie & Krauss 2012), and highlight the importance of using high genetic diversity in the seed pool used for restoration to avoid strong founder effects. Thus, we suggest the use of seeds from different sources to produce seedling for restoration areas. We also suggest that large and well-conserved remnants as main sources of seeds, as they may have smaller effect of inbreeding on effective population size.

We also observed the presence of gene flow from neighbour populations, which indicates that it is possible to recover pollination services in restoration plantations. However, the smaller N_e in disturbed and restoration areas reinforce the importance of enhancing connectivity among fragments to create metapopulation dynamics, increasing the effective population size and slowing down genetic drift effects, key issues for supporting population ecological viability and persistence in a changing environment.

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Chapter 4

A genetic approach for simulating persistence of reintroduced tree species populations in forest restoration areas

Abstract

Many plant populations from forests ongoing restoration are small and isolated from neighbouring populations, and are in human-modified landscapes. To mitigate inbreeding depression and genetic drift problems, there are recommendations for seed sampling aiming to introduce populations with high genetic diversity in restoration areas. However, studies validating or testing those recommendations' feasibility are not available, and ecological and financial constraints prevent obtaining the recommended number of seed sources. It is especially difficult to reintroduce as many tropical tree species in restoration sites as found in reference ecosystems, where there are thousands of species, many of which are rare. We present here an individual-based model that allows to evaluate the effect of different levels of initial genetic diversity on the short and mid-terms' population viability. We also present a study case with *Centrolobium tomentosum*, a tropical tree species widely used in restoration projects in the Atlantic Forest of Brazil, to demonstrate the use of this simulation model. Our model can be applied in studies of tree species with different characteristics, from tropical and temperate forests, to assess population persistence in restoration sites. This knowledge can support planning of both restoration projects and management actions, increasing population viability and minimizing costs.

Keywords

individual-based model, ecological restoration, genetic diversity, population viability

Introduction

Ecological restoration is the process by which ecological processes are recovered with functional and resilient communities, and with species that are able to adapt to changing conditions, while delivering ecosystem services (Alexander *et al.* 2011). Restoration is now acknowledged as a global environmental priority and many international initiatives are aiming to restore millions of hectares around the world. The New York Declaration on Forests is one such initiative that has set a goal to restore 350 million hectares of forest landscapes by 2030 (Suding *et al.* 2015). The proposed massive re-conversion of pasturelands and agricultural fields into native forest ecosystems is seen as necessary to mitigate future extinctions debt in regions with high levels habitat loss and fragmentation (Banks-Leite *et al*. 2014). In such conditions, ecological restoration efforts have to support the conservation and reestablishment of populations with enough genetic diversity to persist overtime and to increase landscape connectivity to facilitate plant and animal gene flow in human-modified landscapes (FAO 2015).

In such landscapes, zones selected for forest restoration can become repopulated through artificial establishment of trees (i.e., active restoration) or by natural colonization and secondary succession (i.e., passive restoration; Holl & Aide 2011). Although humans can reestablish native tree species populations in degraded sites, their persistence may be threatened by various factors, e.g. micro-site limitations for seedling regeneration (Bertacchi *et al*. 2016), competition with invasive species (D'Antonio & Meyerson 2002), pollination and dispersal limitation (Dixon 2009), genetic-mediated processes (Thomas *et al.* 2014), and climate change (Harris *et al.* 2006). Most studies on restoration strategies and methods focus on solving immediate problems regarding timing of planting and on short-term persistence (Bertacchi *et al.* 2015). Mid- and long-term interventions however, tend to be more difficult to predict and are less often studied, and can be seen as less financially viable (Hobbs *et al*. 2011), despite their value and importance to long-term conservation (Suding *et al.* 2015). Mid- and long-term sustainability depends on a species' and populations' ability to persist and evolve in response to environmental changes, which depends on intra-specific population level genetic diversity (Young *et al.* 1996; Booy *et al.* 2000). Populations with reduced genetic diversity tend to show reduced fitness, limited potential for adaptation (Reed & Frankham 2003), and have a higher probability of extinction due to diseases or environmental stochasticity (Mills 2013). Thus, using seeds with high genetic diversity in restoration projects is

one of the main strategies for effective restoration and successful persistent conservation in the face of environmental change.

Genetic issues have been mostly considered in active forest restoration, where populations and mother trees can be selected for seed harvesting (Bozzano *et al.* 2014; Basey *et al.* 2015). In general, restoration practitioners aim to increase the genetic viability (i.e. reduce extincion risk) of reintroduced populations by using seeds from local populations to increase the chances of having local adaptations to the environmental conditions where restoration will be carried out. Also, many mother trees are also targeted to maximize the genetic diversity of the founding population (Lesica & Allendorf 1999; Hufford & Mazer 2003; McKay et al. 2005). However, even projects established with seedlings produced according to a well-planned program of seed collection are generally small. Thus, these populations are subject to the same risk of loss of genetic diversity as populations that have been reduced by habitat loss and fragmentation (Sezen *et al.* 2007; Chazdon 2014). In all these situations, a reduction in heterozygosity and allelic diversity over subsequent generations is expected in tree populations from restoration sites. The smaller the population, the faster we expect to observe the negative effects of genetic drift on genetic diversity (Mills 2013). The loss of genetic diversity in these populations associated with inbreeding depression increases the risk of extinction (Frankham *et al.* 2005) and, consequently, restoration failure.

This issue has worried researchers and practitioners and there are different recommendations for the minimum population size necessary to avoid inbreeding depression in the context of restoration actions in both the short-term (5 generations) and long-term (perpetuity). An effective population size (N_e) of 100 is considered necessary for short-term conservation and $N_e = 1000$ is considered necessary for long-term conservation (Frankham *et al.* 2014). For restoration purposes, it has been suggested as a general rule that seeds should be sampled from at least 30 trees (Sebbenn 2006) or 50 trees (Basey *et al.* 2015) to recover genetic diversity and plant populations with high enough effective population size ($N_e \geq 100$). These recommendations were based in population genetics theory; however there is a lack of studies validating or testing their feasibility. For example, in tropical forest restoration efforts, where over 100 native tree species have been reintroduced in restoration plantations (Rodrigues *et al.* 2011), reaching this minimum number of mother trees per species (i.e., 30-50) can be difficult. There are constraints in both ecological (limited number of mother trees of low density species in fragmented landscapes) and financial

terms (time and resources spent to find numerous mother trees and collect their seeds) (Brancalion *et al.* 2012).

Additionally, it is challenging to undertake experiments to test the effects of different levels of initial genetic diversity on the viability of tree species populations reintroduced in restoration areas, due to financial costs, long periods of time required, and difficulty to control for other potentially confounding environmental factors. Moreover, many particularities of restoration make this decision more complicated. Restoration fragments may have different levels of isolation from other populations, which also vary among species in a fragment, because of different abilities of pollinators and seed dispersers to mediate gene flow (Rudnick *et al.* 2012).

The use of computer models is one potential solution for this problem (Epperson *et al.* 2010). The process through which we evaluate data and models of populations to estimate likelihoods of population persistance over an arbitrary amount of time is called population viability analysis (PVA) (Boyce 1992). Currently, there is an unfortunate lack of programs that simulate the population dynamics of tree species, which have long life spans, and overlapping generations, and that also model spatial and temporal genetic variation. Also, most PVAs assessments ignore or inadequately model genetic factors as the effect of inbreeding depression on total fitness (Frankham *et al.* 2014).

In this study we describe a novel individual-based model to simulate the spatio-temporal genetic and population dynamics of trees in restoration plantations, and show one case study as an example of application of the model. Using this model we evaluate the effect of different levels of initial genetic diversity on the population viability in a short and mid-terms in a tropical tree species.

The model

The description of our model follows the ODD (Overview, Design concepts, Detail) protocol of Grim *et al.* (2006, 2010).

The initial genetic diversity can be represented by the minimum number of mother trees from which is it necessary to collect seeds to establish a restoration plantation. Indeed, this is one of the parameters over which management agencies have the greatest control when designing and

implementing restoration strategies. In this context, the specific questions this model was designed to address are:

1. Do the initial population size and the initial genetic diversity in a restoration project affect the viability of a population in a restoration area?
2. What is the minimum number of mother trees from which seeds should be collected for a restoration forest plantation to ensure sustainable levels of genetic diversity in the restoration population?

Entities, state variables and scales

The entities of this model are individual trees. Each individual is characterized by the following four state variables: (1) spatial location; (2) age; (3) developmental stage (seed, juveniles and reproductive adult); and (4) genotype. Spatial location describes the x and y coordinates in a two dimensional lattice. Tree age can vary from zero to the maximum age that the species can reach and increases by one unit (year) at each time step. The simulations start with all individuals at 3 years old, as in a recent plantation. Seeds have age of zero, and germinated seedlings have age of one. Individuals are considered juveniles before they reach reproductive age and adults are reproductive until the end of life with the same reproduction rate (Fig. 1). For each independent locus, each genotype is composed by a pair of alleles, represented by a pair of integer numbers from one to the maximum number of alleles initially defined. Genotypes represent independent codominant loci, with no linkage disequilibrium (Fig. 2). Any codominant markers can be simulated.

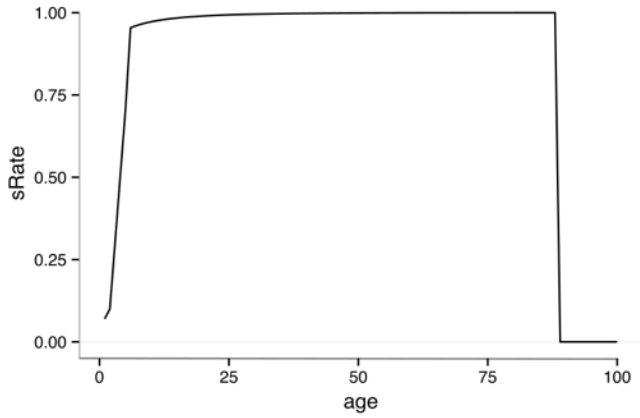

Figure 1. Probability of survival to the next year as a function of age.

The time step in the model is 1 year. The spatial extent of the simulated area consists of a single isolated forest fragment (patch) undergoing restoration. The area shape, always a narrow rectangle, was defined to simulate narrow riparian forests, where there is no light limitation.

Process overview

The model starts by defining the initial parameters, which include age, spatial location, and genotypes of individuals in the initial planted population; the duration (time), and the number of simulation repetitions (rep); the number of loci in the input dataset; the model area dimensions (xDim, yDim); pollen (dPollen) and seed (dSeed) dispersal distances; number of seeds produced by each tree (avSeed); maximum age an individual can reach (maxAge); at what age the individual becomes an adult (adultAge); maximum number of pollen donors for each offspring (maxFathers); germination probability (germ); selection factor (selection); and the time steps when output files should be saved (output_years).

Each cycle starts with the determination of each individual's survival rate, as a function of age. This model consider hermaphrodite tree species, so all individuals can be either mother or father of an offspring, and self-fertilization is possible. The reproduction step starts by defining for each mature tree (i.e., mother), which mature trees (i.e., fathers) are present within the pollen dispersal range. If the number of potential mates is fewer than the maxFathers, all mates contribute to pollination. Otherwise, a number of potential mates equivalent to maxFathers are randomly selected to be pollen donors. By default, potential mates as sampled with a uniform probability distribution, but other probability distributions may be used. Alleles are inherited following Mendelian inheritance.

Seed location is defined by the seed dispersal range and a randomly selected dispersal direction. If a seed falls at the same location of an existing adult or out of the study area, it does not survive. Otherwise, each seed has the predefined initial probability of germination, which can be decreased as a function of its inbreeding coefficient (Richards 2000). In species that produce a large number of seeds from which only a few germinate and survive to juvenile phase, as is the case of most trees, many seeds do not germinate and many seedlings do not survive by chance, so genetic drift has a stronger effect than selection. Thus, selection on germination and survival is not strong enough to purge deleterious alleles efficiently (Keller & Walter 2002). In these situations,

inbreeding depression can affect seed and seedling viability (Naito *et al.* 2005), germination success (Richards 2000), and survival rates (Ishida *et al.* 2005). The assumptions for estimating the inbreeding effects on fitness in this model are: 1) an increase in inbreeding by 10% leads to a reduction in fitness components of 5-10% (Frankel & Soulé 1981); 2) a large number of loci are affected by inbreeding depression (Ayroles *et al.* 2009; Chaves *et al.* 2011); and 3) a sample of loci allows inference about the inbreeding coefficient of a population (Chaves *et al.* 2011). When more precise information about the effects of inbreeding on fitness is available in the literature, they can be incorporated in this model.

If the seed germinates, the new individual is included in the tracking list of active individuals with an age of one. Probability of survival to the next year for both juveniles and reproductive individuals is assessed in each time step as an increasing function of tree age. Dead individuals are removed from the population.

At the end of the time steps defined by the user (output_years), a summary table with information on living individuals (location, genotypes, age and survival probability) is recorded. After the end of simulations, the output datasets are transformed to the FSTAT (Goudet 1995) format and the genetic summary statistics are calculated for all individuals with age ≥ 1, using Hierfstat (Goudet 2005). Summary statistics are: number of individuals, total number of alleles, allelic richness, observed and expected heterozygosity under Hardy-Weinberg Equilibrium (HWE), and inbreeding coefficient, with confidence interval.

Design concepts

1. Basic concepts: The basic principles of this model are that the population is isolated from others (no migration), mutation rates are negligible, individuals mate with others within the pollen dispersal range, which means that the population is not panmictic, and the germination probability is associated with the inbreeding coefficient of each seed. Mortality in all other life-stages is age dependent.

2. *Emergence*: Population size is the total number of individuals with minimum age of 1 year old and is a result of mating rules (see below), survival probabilities and the amount of space available. So the population size is not explicitly modelled and is an emergent property of the simulation. The

frequency and number of alleles change as a result of genetic drift, which is random although influenced by the assumptions of dispersal and overall model spatial scale, thus it is also an emergent property that reflects in the genetic diversity in the population.

3. *Stochasticity*: Randomness in the model operates through mating and survival. Mates are sampled at random from the adults available within the pollen dispersal range. Other than distance from the mother tree, there are no other rules for mating preference. Seed's genotypes are defined by random sample from the parents' genotypes. Each seed's location is defined in the seed dispersal range from the mother tree, according to the rules described in the seed dispersal sub-model, which includes randomness in dispersal direction. Death is represented as a probability that depends on the age of the individual (Fig. 1).

Initialization

Before each simulation, the parameters of the initial population planted in the area must be defined. The saplings genotypes are drawn depending on the number of mother trees from which they descend and may vary from a pool of individuals in HWE to a full sibling pool. The saplings can be planted homogeneously or randomly distributed in the area, or aggregated in one or more clusters.

Input

The input dataset consists of a tabular delimited table with individual location (x, y), genotypes, and age (Fig. 2). There is no limitation for number of individuals and the size of the restoration area.

	x	y	l1_1	l1_2	l2_1	l2_2	l3_1	l3_2	l4_1	l4_2	l5_1	l5_2	l6_1	l6_2	l7_1	l7_2	l8_1	l8_2	l9_1	l9_2	l10_1	l10_2	age
1	214	16	2	1	3	1	5	2	1	2	3	2	4	5	1	3	4	4	1	3	1	3	3
2	737	18	3	5	4	5	3	2	5	4	5	3	3	3	3	1	5	1	1	2	1	2	3
3	2765	13	5	4	3	4	3	1	4	4	4	4	5	2	4	4	2	4	1	4	3	4	3
4	5965	19	3	3	4	3	2	4	4	1	4	4	2	2	1	2	2	5	2	2	4	2	3
5	6072	18	1	2	1	2	4	5	2	5	4	5	2	3	1	1	4	4	5	4	3	2	3
6	3506	9	4	1	5	1	3	4	1	5	1	2	1	4	3	1	5	4	2	4	1	4	3
7	2436	1	1	5	1	5	2	5	3	2	4	4	4	1	1	1	3	2	3	1	2	5	3
8	6541	5	3	5	5	5	2	5	1	3	1	5	5	1	4	4	5	3	1	1	5	1	3
9	5487	13	3	1	5	1	2	1	3	4	1	2	3	5	4	2	3	5	3	5	3	2	3
10	289	12	3	2	1	2	5	1	4	4	2	3	3	1	5	4	5	5	3	5	3	1	3

Figure 2. Input data example with 10 individuals, their location (x, y), genotypes (l_i_1, l_i_2) and age.

Sub-models

The model contains four sub-models: (1) reproduction; (2) seed dispersal; (3) seed germination; and (4) age-specific mortality.

1. Reproduction: This sub-model simulates sexual reproduction among individuals in the pollen dispersal distance range. The model does not simulate asexual reproduction or gene flow from other, non-explicitly simulated populations. Reproductive events occur once a year, all individuals in the adult life stage are considered reproductive, all have the same potential for pollen and seed production, and there is no pollen limitation. The pollen donors are randomly sampled from the pool of individuals in the pollen dispersal distance range. We assumed Mendelian inheritance of alleles, as the model's loci represent independent nuclear genetic marker loci (i.e., no linkage). Selfing was allowed in the simulations.

2. Seed dispersal: We assumed isotropy (no preferential direction for dispersal), so the seed location is defined by the dispersal distance function and a random value from one to 2π radians for the direction. All seeds produced in a year germinate or die, so there is no soil seed bank formation.

3. Seed germination: We modelled the probability of seed germination as a function of inbreeding. Thus, in this sub-model, the probability of germination is estimated using all neutral loci, wherein if the inbreeding coefficient (F_{IS}) is greater than zero, an increase in inbreeding leads to a reduction of germination rate in the scale defined by the selection factor (gRate = g*(1- selection*F_{IS})), where g is the probability of germination with no inbreeding. So, a selection factor of 0.5 means that an increase in inbreeding by 10% leads to a reduction of 5% in germination rate. If inbreeding coefficient is zero or negative, it has no effect on germination rate.

4. *Mortality*: The mortality probability was modelled as an age dependent function. All individuals die when reach the maximum age. For each individual in each time step, a random number is sampled from a uniform distribution between zero and one. If the number is larger than the survival probability (sRate), the individual dies.

Model development

We wrote the program script in R (R Core Team 2015). General model schematic is described in Fig. 3 and the program script can be found in supporting information and also at GitHub (https://github.com/sujiips/Restoration_PVA.git).

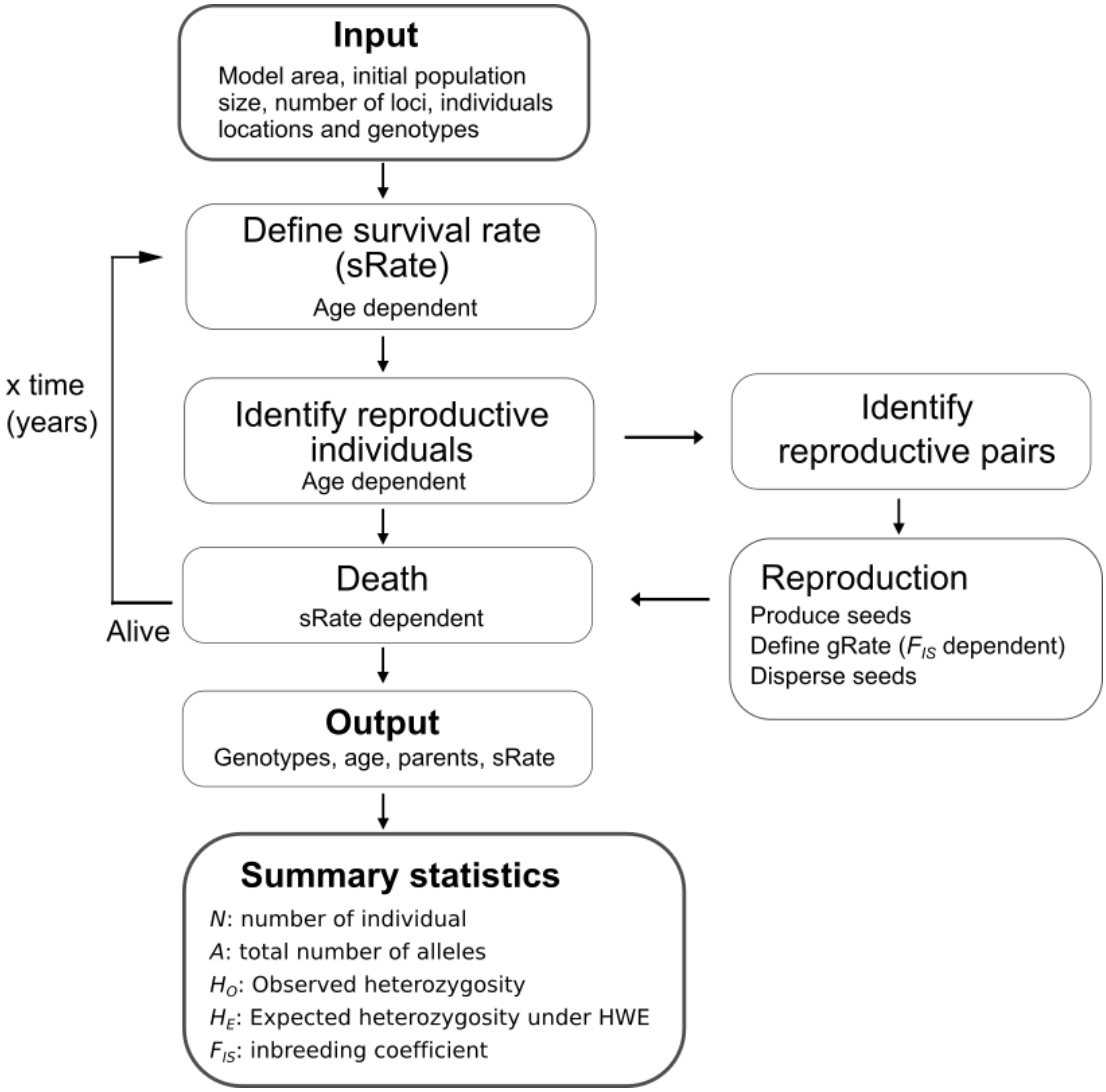

Figure 3. Model flow chart.

Sensitivity analysis

Sensitivity analyses were performed to quantify the relative importance of each model process on simulation results and to how uncertainty in parameter values affects the model reliability. All simulations for sensitivity analysis used a restoration area of five hectares with a

presumed seed source of a large population and using reference parameter values from *Centrolobium tomentosum* (Fabaceae), a tree species from an intermediate stage of ecological succession from a tropical forest. Each simulation was run for 500 time steps and each condition was repeated 250 times. The number of repetitions was defined to minimize the effects of stochastic variation on the estimated parameter mean (Fig. 4).

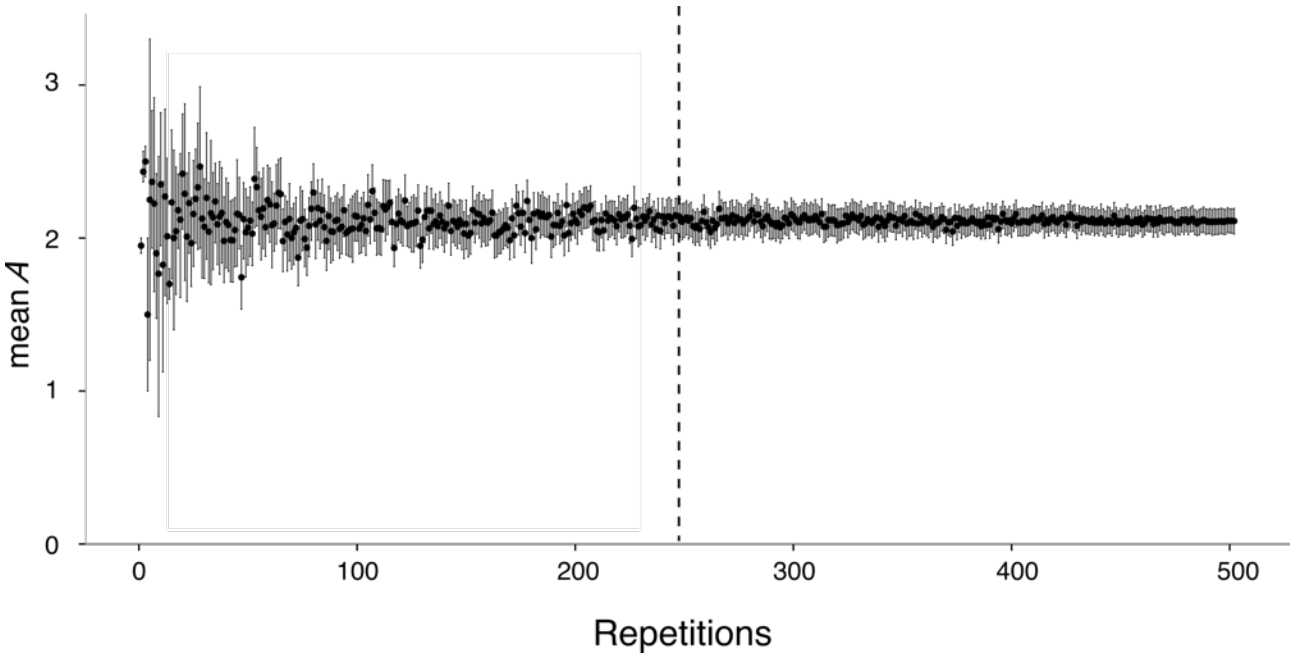

Figure 4. Mean values for the mean number of alleles in the population (*A*) for each number of simulation repetitions. The bars represent the 95% confidence intervals and the scattered line indicates 250 repetitions.

We performed a local sensitivity analysis (Railsback & Grimm 2012), varying the parameter values one at a time. The range to vary the parameter values were defined following the rule of thumb (+/- 5%), except for some parameters, for which a 5% variation was too small to produce noticeable response. In this situation, a higher percentage of variation was selected.

Model application

C. tomentosum (Fabaceae) was selected as the species for the exposition of this simulation model because this is a tree species widely used in restoration projects for which demographic and genetic data are available. This species is frequently used for forest restoration because it is a typical gap species, that reproduces once a year, with relatively fast growth, and has symbiotic associations with nitrogen fixation microorganisms (Carvalho 2005; Pagano 2008). This species has

a wide range of distribution over the Atlantic Forest and some parts of Cerrado, global Biodiversity Hotspots.

We sampled this species in a 20-ha restoration plantation established in a riparian buffer around a reservoir from 1988 to 1990, in Iracemápolis municipality, São Paulo State, in the Atlantic Forest region of southeastern Brazil (W 47º 31' 09", S 22º 34' 36") (Brancalion *et al*. 2014). The restoration model in this area was the use of a combination of species from different successional stages in modules of planting (6 pioneers and 2 early secondary, 1 late secondary or climax). This forest patch was also established with high species diversity (140 species), most of them native (77%), chosen from available seedlings in commercial sources (Rodrigues *et al*. 1992).

We simulated restoration areas with different initial conditions to determine the effect of the following factors on genetic diversity and short and mid-terms population viability in restoration zones: 1) initial population size; 2) initial genetic composition (number of alleles and genotypic frequencies).

All the simulated patches were riparian areas (long and narrow), with 30 m width. We simulated areas with 5 ha, 10 ha and 20 ha. The initial population size was dependent of the patch area, because the current recommendations for forest restoration is to plant 20 ind/ha. Simulations were run over 30, 50, 100, 250 and 500 years. The initial genetic composition was determined by the seed source characteristics:

1. One isolated tree: seeds produced from 100% selfing;
2. Partially isolated trees: patches with five and 10 trees, seeds collected from one to 10 mother trees and different number of pollen donors;
3. Forest fragment: patch with a large population, seeds collected from one to 10 mother trees and different number of pollen donors.

Maximum tree age is limited to 89 years. This parameter was estimated according to Laurance *et al*. 2004, using mean values of growth rate and functions based on both canopy and emergent trees. Growth rate for *C. tomentosum* was estimated using diameter measures of trees from restoration areas with different known ages (Silva 2013). The probability of surviving to the next time step was estimated using a demographic study of adult individuals from a natural remnant forest fragment (Barreto 2015; Silva 2013) and it follows a Weibull distribution. As there were no available data for seedlings and saplings survival rate, a linear function was utilized. The function

describes an increase of 0.2 in survival rate every year, from one year old until the age of six, for which there are information about survival (Fig. 1).

As *C. tomentosum* is a hermaphrodite species, all individuals can be either mother or father of an offspring, and self-fertilization is also possible. The main pollinators are large bees with long distance flight capacity. The maximum distance of pollen flow (1000 m) was based on information on flight patterns and mean maximum flight distances of large bees, that are the main pollinators of *C. tomentosum* (Pasquet *et al.* 2008; Hagen *et al.* 2011). The pollen donors are randomly sampled from the pool of individuals in the pollen dispersal distance range, following the pollinators' foraging behaviour (Keasar *et al.* 1996). The species has a mixed mating system, and the maximum number of pollen donors for each mother tree is 20 trees (Chapter 2).

C. tomentosum has one or two seeds enclosed in a large (~ 9 g each) thorny samara (winged fruit), that does not remain in permanent seed banks and are dispersed by wind for short distances (at most 100 m away from the mother tree, following a Weibull distribution),so most seeds fall under the canopy of the mother tree (Cavalho 2005; Aidar & Joly 2003; Chapter 2). The germination probability is 0.7 (Carvalho 2005). The parameter values used for the simulations are in Table 1.

To produce the input datasets, we first simulated a large original population (N = 1000), with all loci in Hardy-Weinberg Equilibrium (HWE), and the same number of loci and alleles that would be used in the simulations (10 loci, 5 alleles). From this original population, we sampled the individuals for the simulations with initial genetic composition in HWE. For the simulations with saplings from one mother tree, we sampled one individual from the original population to be the mother. This mother was the source of the first allele of each locus of each sapling used in the plantation. The second allele of each locus was randomly sampled from the original population allele pool, simulating panmixia. For the simulations with saplings from more that one mother tree, we applied the same procedure as for one mother, but using more sampled individuals as mothers.

Table 1 – Parameters and values of the model

Parameter	Value/ Distribution	Reference
Reproduction		
Sexual maturity age	age = 10 years	Brancalion (comunication)
Maximum pollen dispersal distance	$D_{pollen} \leq 1{,}000$ m	Pasquet *et al.* 2008; Hagen *et al.* 2011
Pollen donors	$n \leq 20$	Chapter 2
Seed dispersal		
Maximum seed dispersal distance	$D_{seed} \leq 100$ m	Chapter 2
Seed dispersal function	Weibull distribution (k = 0.6942; λ = 81.4807)	Chapter 2
Germination		
Germination probability function	$F_{IS} \leq 0$, g = 0.7 $F_{IS} > 0$, g = 0.7*(1 - 0.5*F_{IS})	Carvalho *et al.* 2005; Frankel & Soulé 1981; Richards 2000
Mortality		
Maximum age	age = 89 years	Laurance *et al.* 2004; Silva 2013
Survival rate	age = 1, sRate = 0.1 1 > age ≤ 5 years, sRate = 0.2*age - 0.3 age > 5 years, sRate = Weibull function (k = 0.7631, λ = 7.8216) age > 89, sRate = 0	

Results

Sensitivity analysis

Our results indicated that our model is not overly sensitive to any of the parameters tested (Table 2). Each parameter had different effects on mean number of alleles (*A*), inbreeding coefficient (F_{IS}) and proportion of extinct populations. The parameters with the strongest effect were maximum age that a tree can reach, germination rate, selection pressure value, and average number of seeds produced by each tree on the number of alleles (Table 2).

Table 2. Values for number of alleles (*A*), inbreeding coefficient (F_{IS}) and proportion of extinct populations estimates in the local sensitivity analysis and the sensitivity coefficient (S).

			A		F_{IS}		Proportion of extinct populations	
Variable	Value	Variation	*value*	S	value	S	value	S
Reference condition	*		1.83		0.396		0.480	
Adult age	5 yrs	- 50%	2.52	1.37	0.204	-0.383	0.388	-0.184
Adult age	15 yrs	+ 50%	1.66	-0.35	0.473	0.155	0.676	0.392
Pollen dispersal	500 m	- 50%	1.79	-0.08	0.458	0.125	0.560	0.160
Pollen dispersal	1500 m	+ 50%	2.00	0.33	0.354	-0.084	0.532	0.104
Maximum number of pollen donors	15	- 25%	1.88	0.18	0.362	-0.136	0.484	0.016
Maximum number of pollen donors	25	+ 25%	1.95	0.48	0.347	-0.194	0.528	0.192
Seed dispersal	50 m	- 50%	2.98	2.30	0.097	-0.597	0.136	-0.688
Seed dispersal	150 m	+ 50%	1.55	-0.56	0.531	0.271	0.676	0.392
Germination rate	0.6	- 14%	1.50	-2.37	0.592	1.376	0.752	1.904
Germination rate	0.8	+ 14%	2.52	4.80	0.168	-1.594	0.260	-1.540
Selection pressure value	0.475	- 5%	1.93	2.01	0.358	-0.751	0.448	-0.640
Selection pressure value	0.525	+ 5%	2.01	3.62	0.321	-1.502	0.520	0.800
Maximum age	85 yrs	- 5%	1.71	-2.81	0.424	0.641	0.592	2.492
Maximum age	93 yrs	+ 5%	2.14	6.86	0.272	-2.758	0.440	-0.890
Average number of seeds per tree	74	- 5%	2.04	3.58	0.346	-0.878	0.436	-0.770
Average number of seeds per tree	67	+ 5%	1.595	-5.549	0.512	2.723	0.576	2.240

Reference values: Adult age = 10 years; Pollen dispersal = 1000 m, Maximum number of pollen donors = 20, Seed dispersal = 100 m, Germination rate = 0.7, Selection pressure = 0.5, Maximum age = 89 years, Average number of seeds per tree = 70.

Model application

Both initial population size and initial genetic composition had an impact on population viability and long-term maintenance of genetic diversity (Table 3). We observed that larger populations resulted in smaller effects of genetic drift, measured here as loss of total number of alleles (Fig. 5a). Especially in larger initial populations (200 and 400 individuals), an increase in the number of pollen donors reduced the rate of loss of alleles (Fig. 5a). Initial genetic composition had a stronger effect on the inbreeding coefficient in populations from the small areas (Fig. 5b). Sampling seeds from only one isolated tree resulted in a larger increase in inbreeding and in stronger bottleneck effects. Selection against overall homozygosity at the germination phase kept the inbreeding coefficient small or moderate, even with fixation of alleles at some loci (Fig. 5b). Populations in smaller areas that were founded by seeds from only one isolated mother tree had a greater probability of extinction (Fig. 5c).

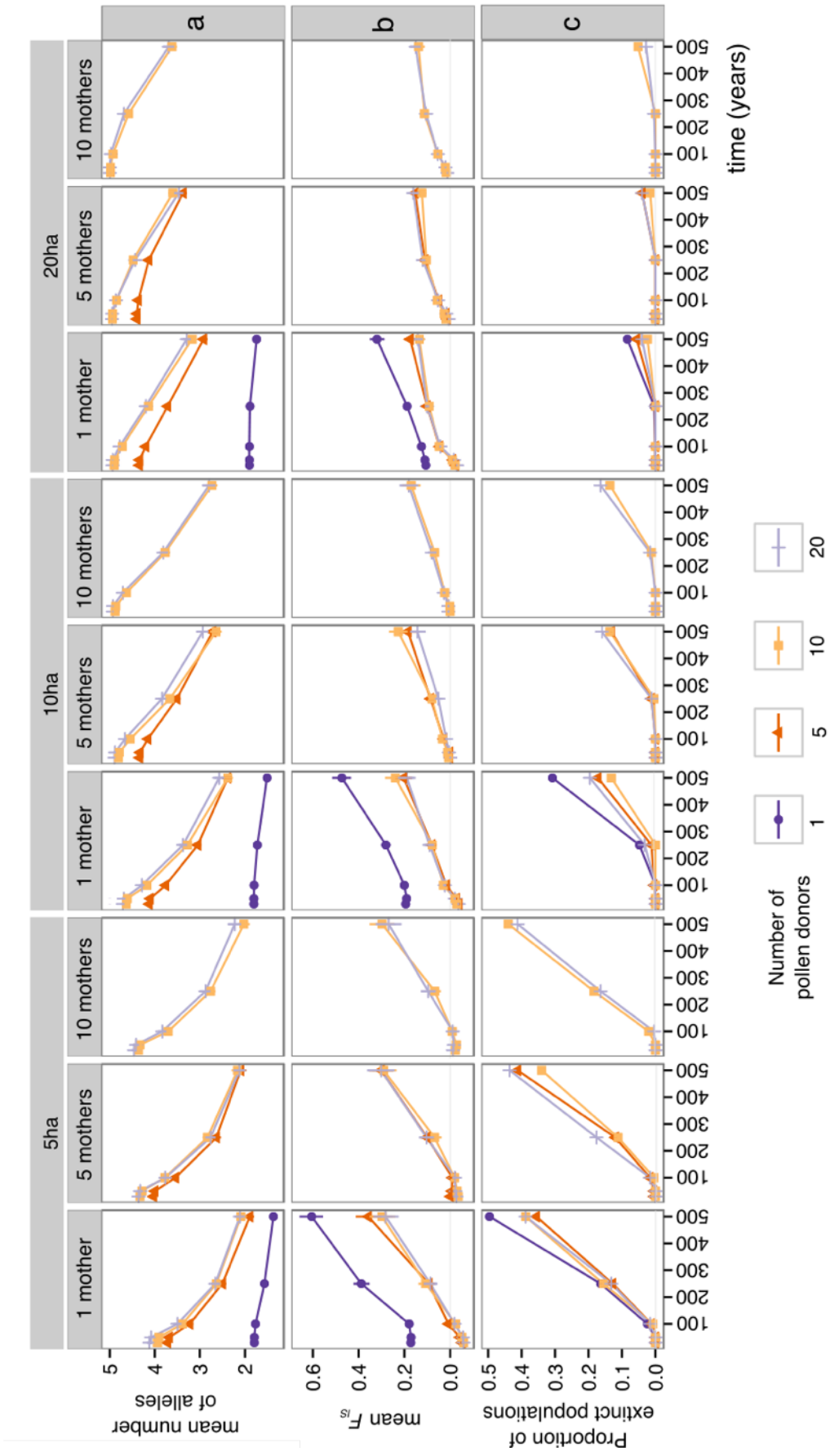

Fig. 5. Effects of initial genetic diversity and initial population size on: a) mean number of alleles observed in each population after different time spans for each treatment; b) mean inbreeding coefficient (F_{IS}) observed in each population after different time spans for each treatment; and c) proportion of populations that became extinct after each time span for each treatment.

Discussion

This model is a tool for understanding the effects of initial genetic diversity on the viability of populations in forest restorations. It can be applied in studies of tree species with different characteristics, from tropical and temperate forests, as observed in the case study.

The use of stochastic space–time simulations has many advantages over mathematical models, because we can incorporate stochasticity, individual variation, adaptive traits, and other complexities important in biological systems (Epperson 2010). Individual-based models may be particularly useful for planning reintroduction and conservation of endangered species in restoration areas. Habitat loss and population disruption by fragmentation are two of the main causes of species extinction (Fahrig 1997), so the introduction of endangered species in restoration projects can be a complement to the species conservation in natural remnant areas. Nevertheless, endangered species are usually found in small population sizes and in low densities, which hampers seed sample both for ex-situ and in-situ conservation. In addition, sometimes it is unfeasible to find the minimum number of source trees previously suggested by the literature (Basey *et al.* 2015). This model is a valuable tool for decision-making since it may help restoration and conservation practitioners to design seed sampling for introduction of endangered species in restoration areas. For most tree species, some of the information required for using our model is not available, but the availability of datasets on native species autoecology and population structure is growing fast (Kattge *et al*. 2011; Canhos et al. 2014; de Lima *et al.* 2015). In addition, some information can be extrapolated from species with similar ecological and demographic characteristics. In addition, there is more information on the literature for key species, and for threatened species (Sousa-Baena *et al*. 2013).

This model accounts for overlapping generations and effects of inbreeding depression, which are present in many tree species (Angeloni *et al.* 2011). The effects of inbreeding depression were summarized in this model in the germination process, that is when the negative effects of inbreeding are strong and were more carefully measured (Ishida *et al*. 2005; Naito *et al*. 2005; Chaves *et al.* 2011). Although there is evidence that inbreeding may reduce survival and reproduction in different life stages (Hufford & Hamrick 2003; Naito *et al.* 2005), there is a lack of studies testing the magnitude of the effect on later life stages. When this information become available in the literature, we can incorporate the effects of inbreeding depression on different life stages.

This model was designed for simulations of populations in the short and mid-terms (tens of generations), because it does not account for mutation and for changes in selective pressures over time. It also focuses on only one isolated population, so it is not possible to evaluate effects of inter-population gene flow. These limitations may be overcome using the results obtained from our model as input or assumptions for other simulation programs that account for mutation, migration and natural selection in changing environments. A list of such programs can be found in Epperson *et al.* (2010).

For *C. tomentosum*, if the restoration area is long and narrow, as in riparian forests, the initial population size is an important characteristic in order to consider to reduce the risk of genetic bottlenecks, i.e. abrupt changes in allele frequency and loss of genetic variation (Allendorf *et al.* 2013). In general, the smaller the area, the smaller the initial population size and the greater should be the concern with the genetic composition of the seed pool to maintain high genetic diversity and increase the long-term viability. Consequently, small restoration patches may not sustain genetic conservation of many tree species, so ecological prioritization models have to determine which species should be used according to their conservation value, importance for provisioning ecosystem services, supply timber and non-timber forest products, and other targeted functions in restoration. In all sizes of restoration area, planting seeds from only one isolated tree results in a very strong bottleneck effect, higher extinction rate and genetic diversity loss. This knowledge may support planning of both restoration projects and management actions, increasing population viability and minimizing costs.

Simulation studies have been performed for diverse applications in forest restoration projects, such as to assess the impact of ecophysiological parameters on species resilience of forest stands (Pietsch & Hasenauer 2002); to understand the influence of management on forest structure over time (Corvington *et al.* 2001); and to predict habitat quality in restoration plantations (Pausas *et al.* 1997). Simulations are a particularly useful tool for examining population persistence in forest restoration in situations where empirical manipulation of the system is either too costly financially, or simply limited to the rarity and conservation status of the taxa being examined. Nonetheless, integration of simulation and empirical studies will further serve to inform decision making processes and ultimately improve the probability of success and long term persistence of restoration efforts. As far as we are concerned, this is the first model to simulate population persistence in

restoration sites with a population biology and genetic approach, and has a great potential to support seed collection, restoration implementation and adaptive management, but requires further tests, adaptations, and improvements to better address specific goals and conditions of restoration projects.

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General discussion

Overall, our results indicated that it is possible to recover genetic diversity of *Centrolobium tomentosum*, a allogamous tree species, in ecological restoration areas. However, it is fundamental to guarantee high diversity in the seed pool used in for restoration, avoiding founder effect, and to restore connectivity among fragments to increase effective population size and reduce the impacts of genetic drift.

C. tomentosum is an outcrossing species ($\hat{t_m}$ = 0.98), with limited seed dispersal and long pollen dispersal distance. These characteristics, associated with self-compatibility (Aidar 1992), relatively fast growth (Carvalho 2005), and symbiotic associations with nitrogen fixation microorganisms (Pagano 2008) make this species very useful in restoration projects. This species' seeds probably will not disperse naturally to distant fragments, but if they are actively planted in a restoration area, they will grow fast and reproduce even if pollination services are not yet well recovered. When pollinators are present, the genetic diversity in the population can be maintained by cross-fertilization, and gene flow among populations can increase diversity and slow down genetic drift effects.

The low gene flow by seed dispersal causes a strong spatial genetic structure in the population, when we analyze only DNA maternally inherited. This low gene flow by seeds is partially compensated by the pollen dispersal kernel (exponential power distribution), which indicates that a large amount of pollen is dispersed through long distances, and the high frequency of outcrossing in the species. The spatial genetic structure estimated with nuclear DNA, inherited from both parents is weaker and similar to other species with long distance gene flow (Vekemans & Hardy 2004; Hardy *et al.* 2006; Dick *et al.* 2008).

We detected a larger number of haplotypes from chloroplast DNA in both restoration areas, when compared to natural remnants. This indicates that the seedlings used in the restoration projects came from different provenances, probably from more than one population. This result is similar to the observed for two tree species from Cerrado, in which the high allelic richness indicated that seeds for restoration were sampled from different fragments (Rodrigues 2013). The high genetic diversity in the seed pool resulted in weak founder effect, which is reflected in the high levels of allelic richness and heterozygosity observed in microsatellite markers in adult individuals. We also observed private alleles in juveniles in restoration areas, which is an evidence of pollen flow from

neighbouring areas. This indicates that pollination services are being also recovered in the areas and that pollinators may connect populations from different patches.

We also found similar levels of inbreeding comparing restoration areas populations to natural remnants. The analysis of individuals pooled according to their life stages (juvenile and adult) showed that the mating system is probably not being affected by disturbances as fragmentation. It also shows that there is no evidence of pollination service deficit in restoration areas. The similar patterns of spatial genetic structure corroborates these results. Other studies of genetic diversity and inbreeding levels in other species in the same areas showed the same patterns as the presented in this study. A study that investigated the genetic diversity in monospecific stands of restoration areas found lower genetic diversity than in natural remnants (Neto et al. 2014). Estimates of representativeness of our samples showed that inbreeding and coancestry were not affecting negatively the effective population size. This indicates that these populations have high probability of maintenance of genetic diversity over generations (Vencovsky & Crossa 2003; Raposo et al. 2007).

Using crossing rates and coancestry information, we estimated the minimum number of trees from which sample seeds for restoration purposes. Our results indicate that sampling seed from 39 trees would produce a seedling pool with effective population size of 100, the recommended for short-term conservation (Frankham 2014). This number agrees with other studies that support restoration planning (Sebbenn 2006; Basey *et al.* 2015).

Although the ideal situation is to restore populations with a large effective population size, to increase long term viability (Bozzano et al. 2014), in some cases it is not feasible or possible. Many tree species from tropical forests are rare or occur in low densities (Hubbell and Foster 1986; Slik et al. 2015), which hampers the achievement of the minimum number of seed-trees. Besides, some restoration areas are very small (< 10 ha) and may not have enough space for an effective population size of 100. Our individual based model can support management and restoration planing in these situations. It is possible to simulate populations in areas of different sizes, and with different initial genetic diversity to assess the effects of these parameters on population viability. This model accounts for overlapping generations and effects of inbreeding depression, which are present in many tree species (Angeloni *et al.* 2011), and are not yet well explored in other simulation programs (Epperson et al. 2010).

We performed a case study with *C. tomentosum* to validate the model and test the program. The results obtained agreed with to the expected, according to population genetics theory. Analyzing fragments from five to 20 ha, we observed that initial genetic diversity levels have stronger effect on populations from small areas. Different levels of initial genetic diversity were translated in the model as the number of seed-trees and pollen donors that contribute to the seed pool used in the restoration. We observed that using seeds from one isolated tree has strong negative impacts on population viability. We also observed that there is no difference on viability sampling seeds from 10 or 20 trees, if the number of pollen donor is large.

This model can be used for different species, with different ecological and demographical characteristics to better understand the effects of initial genetic diversity on population viability. Simulating different populations, and a large range of area sizes and genetic diversity can lead to generalizations that may be used to support decision-making in restoration plans.

Conclusion

Centrolobium tomentosum is an allogamous species, with biparental inbreeding, probably due to the spatial genetic structure. The seed dispersal is limited to tens of meters, with very rare longer distance dispersal, due to the large size and weight of the fruits (samaras). The long flight capacity of the pollinators (large bees) enables that a large proportion of pollen is dispersed over hundreds of meters, and some may be dispersed over thousands of meters. Both seed dispersal and pollen flow have effect on spatial genetic structure. The long distance pollen dispersal partially compensates gene flow dispersal limitation by seeds.

It is possible to recover high genetic diversity in populations planted in restoration areas, since the seeds used in the plantation have high genetic diversity. The maintenance of genetic diversity over generation may be enhanced with large effective population sizes and gene flow among neighbouring fragments. It is also possible to recover inbreeding levels, providing the pollination service is also restored.

The model developed in this project can be used to simulate populations with different characteristics and lead to generalizations. This is especially useful for restoration and management planing in tropical forests, where there are many species still poorly understood.

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Supplement

Published articles

1. Zucchi MI, Atanasio CM, Sujii PS. 2013. Conservação de espécies da mata atlântica com potencial medicinal. Pesquisa & Tecnologia, 10(1).
2. Siqueira MB, Sujii PS, Bajay M, Grando C, Schwarcz K, Macrin C, Zucchi MI. 2013. How can molecular ecology contribute to forest restoration?. Journal of Biotechnology and Biodiversity, 4(4): 316-321.
3. Sujii PS, Silvestre E de A, Grando C, Viana JPG, Siqueira MVBM, Salazar VLP, Zucchi MI. DNA e Meio ambiente, um vídeo que ilustra como a genética pode ajudar na conservação da biodiversidade. Genética na Escola.

CONSERVAÇÃO DE ESPÉCIES DA MATA ATLÂNTICA COM POTENCIAL MEDICINAL

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A Mata Atlântica é um dos biomas mais importantes e mais ricos em biodiversidade do planeta, entretanto, é um dos mais ameaçados e muitas espécies de plantas e animais estão em extinção.

Ocorre principalmente junto ao litoral brasileiro, do Rio Grande do Sul ao Rio Grande do Norte, chegando até o interior do país, abrangendo ainda parte da Argentina e do Paraguai. É formada por diversos tipos de vegetação nativa, sendo estas: as Florestas Ombrófilas, Florestas Estacionais Deciduais e Semideciduais, os Mangues, as Restingas e os Campos de Altitude.

Nela vivem milhares de espécies de plantas e animais, algumas são endêmicas, isto é, somente ocorrem na Mata Atlântica, em nenhum outro lugar do mundo. Além disso, este ecossistema contribui para a preservação de rios e nascentes de sete das nove bacias hidrográficas brasileiras, para o controle do clima e é fonte de alimentos entre outros (A MATA ATLÂNTICA, 2012).

A destruição da Mata Atlântica é um dos mais alarmantes problemas de conservação ecológica do mundo. Para preservarmos esse bioma precisamos conservar as florestas existentes, juntamente com sua diversidade de espécies e também restaurar as áreas já degradadas. Além da diversidade de espécies, também é importante conservar a

diversidade genética, ou seja, garantir que os organismos de uma espécie que vivem em um local não sejam todos idênticos. Desse modo, aumentamos as chances da espécie resistir a mudanças ambientais e a doenças, evitando a extinção.

O Brasil é considerado um dos países com maior diversidade vegetal, abrigando 55 mil espécies catalogadas, sendo que 4 mil espécies vegetais são utilizadas com fins medicinais, resultado da observação e manejo da flora por povos tradicionais. No entanto, a conservação e a exploração sustentável desses recursos genéticos dependem dos estudos sobre a diversidade genética (ZUCCHI, 2009).

Os objetivos desta pesquisa são: estudar a diversidade genética de quatro espécies de árvores nativas da Mata Atlântica com potencial medicinal; e comparar a diversidade das espécies alvo em áreas de matas nativas e áreas em processo de restauração florestal, para orientar ações efetivas para conservação destas árvores nativas.

As espécies alvo deste estudo são: o Araribá, a Cabreúva, a Guaçatonga e o Pau-jacaré. Os fragmentos naturais são a Mata de Santa Genebra e a Mata Ribeirão Cachoeira em Campinas (SP), Estação Ecológica de Caetetus em Gália (SP). Os fragmentos restaurados ficam em Cosmópolis (SP) e Iracemápolis (SP). Estudos como este fazem parte de um conjunto de ações que contribuem para a preservação da natureza, visando a sustentabilidade do nosso planeta. Esta iniciativa faz parte do programa BIOTA financiado pela FAPESP (processo 2011/50296-8).

Dentre as diversas ações do projeto envolvendo a linha de estudo da diversidade genética, temos também o foco na *educação ambiental* em que se trabalha em colaboração com as escolas de Piracicaba com atividades didáticas além de plantio de mudas de espécies nativas. Um *folder* com informações sobre as espécies estudadas neste projeto foi elaborado e encontra-se disponível no site do projeto (www. genomicadaconservacao.com.br/folder). Todos os resultados obtidos nesta pesquisa estão sendo divulgados no site www.genomicadaconservacao.com.br

A seguir apresentamos as principais características das espécies alvo deste trabalho. É importante ressaltar que nenhuma planta deve ser utilizada como medicamento sem recomendação médica, uma vez que podem existir efeitos tóxicos, dependendo da forma como a mesma é utilizada.

Araribá (*Centrolobium tomentosum* Guillem ex. Bentham)

As árvores desta espécie pertencem à família Fabaceae, podem alcançar até 35m de altura e 1m de diâmetro (Fig. 1A). Sua madeira é utilizada na construção civil e naval e na carpintaria. Ocorre na Mata Atlântica dos Estados de Goiás, Minas Gerais, Mato Grosso do Sul, São Paulo e Paraná.

Estudos revelaram a presença propriedades medicinais, ainda em estudo, com possível atividade antialergênica e antiinflamatória e compostos com atividade anti-leishmania (ARAUJO et al., 1998).

Cabreúva (*Myroxylon peruiferum* Linnaeus, Carl von f.)

É uma espécie da família Fabaceae que pode alcançar até 20m de altura (Fig.1B). Sua madeira é utilizada na fabricação de móveis, na construção civil, entre outros. Seu óleo essencial é utilizado pela indústria cosmética. Ocorre em todo Brasil, principalmente na Mata Atlântica, nos Estados do Espírito Santo, Minas Gerais, São Paulo, Paraná, Mato Grosso e Goiás.

Da cabreúva é extraído o bálsamo-do-Peru, empregado na medicina popular como analgésico para infecções do trato urinário e respiratório, diabetes e contra a micobactéria gram-negativa *Helicobacter pylori*, além de ser usado pela indústria cosmética e de perfumaria. De suas folhas foram isoladas substâncias que apresentaram atividade frente à *Mycobacterium tuberculosis*, *M. avium* e *M. kansasii* (CARVALHO, et. al.; 2008). Também há registro de atividade de extrato da espécie contra *Streptococcus pyogenes*, *Shigella sonnei* e *Staphylococcus aureus* (GONÇALVES, et. al.; 2005).

Guaçatonga (*Casearia sylvestris* Swartz)

Conhecida também por Erva-de-lagarto e Café-bravo, pertence à família Salicaceae e pode atingir até 6m de altura (Fig.1C). Tem importância para o repovoamento de áreas degradadas. Pode ser usada para arborizar a cidade, porque tem tamanho médio e raízes profundas, portanto não estragam as calçadas.

Sua madeira pode ser utilizada como lenha, na construção civil e na marcenaria. Ocorre em todo o Brasil, em praticamente todas as formações florestais. A espécie possui várias substâncias de interesse. *Casearia sylvestris* Sw. (Salicaceae), ou guaçatonga, é uma espécie vegetal de ampla ocorrência no Cerrado e na Mata Atlântica que apresenta diversas propriedades medicinais. Os diterpenos clerodânicos produzidos por esta espécie têm despertado o interesse da indústria farmacêutica, e alguns deles (denominados casearinas)

foram patenteados por pesquisadores japoneses como agentes antitumorais (ITOKAWA et al., 1990).

Pau-jacaré (*Piptadenia gonoacantha* (Mart.) J. F. Macbr.)

Planta da família Fabaceae, alcança de 10 a 30m. A árvore cresce rápido, inclusive em solos pobres e degradados, por isso é muito usada na recuperação florestal. Além desse uso, serve também para a produção de carvão, de lenha com aroma agradável e de mel. Ocorre principalmente em regiões de Mata Atlântica.

É pouco frequente em zonas de transição com Floresta das Araucárias e é rara no Cerrado. Ocorre nos estados do Espírito Santo, Minas Gerais, Rio de Janeiro, São Paulo e Paraná. O tronco destas árvores se assemelha com um jacaré (Fig. 1D).

Estudos fitoquímicos da casca, dos galhos e das folhas do Pau-jacaré, elaborados por Carvalho et al. (2010), encontraram diversas classes de componentes químicos, como o aspefenamato, terpenóides e flavonóides. Esta última classe é reconhecida pelos efeitos antiinflamatórios e antialérgicos, e, para *P. gonoacantha*, destaca-se a apigenina, que atua no combate ao câncer, o que evidencia o alto potencial medicinal da espécie.

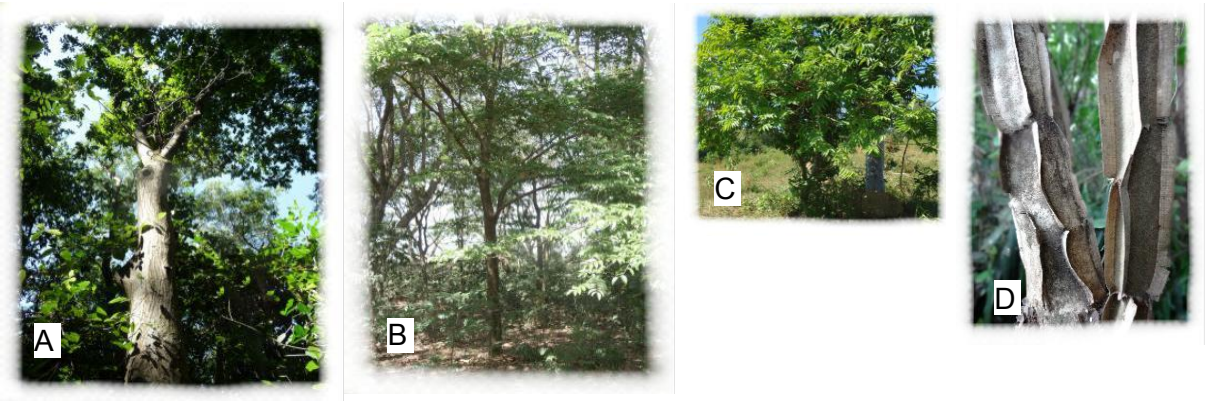

Figura 1 – A- árvore do Araribá, com altura expressiva; B – a Cabreúva; C- a Guaçatonga; D -o caule característico do Pau-Jacaré.

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How can molecular ecology contribute to forest restoration?

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ABSTRACT

The advance of scientific knowledge in various areas of molecular ecology has allowed the adoption of new strategies, particularly in forest restoration. The fusion of multidisciplinary areas and the implementation of management methodologies in order to get better results in forest restoration are current realities. In order to review the main ideas about the role of molecular techniques in the service of ecology restoration, this paper outlines how forest recovery can benefit from genetic and genomic plant population studies. The next challenges in conservation genetics can be brought by the quest for more efficient forest restorations from the point of view of biodiversity as well as the ecological dynamics as a whole. It is believed that in the coming years we will observe integrated strategies in molecular ecology with specific methodologies for restoration in tropical forests.

Keywords: *forest restoration, conservation genetics, molecular ecology, population genetics.*

Como pode ecologia molecular contribuir para a restauração florestal?

RESUMO

O avanço do conhecimento científico nas várias áreas da ecologia molecular tem permitido que novas estratégias sejam adotadas, nomeadamente, na disciplina de restauração florestal. A fusão de áreas multidisciplinares e a implementação de metodologias no sentido de buscar melhores resultados na restauração florestal são realidades atuais. Objetivando rever as principais ideias sobre o papel das técnicas moleculares a serviço da restauração ecológica, o presente trabalho traça como a recuperação das florestas pode ser beneficiada pelos estudos de genética e de genômica populacional de plantas. Os próximos desafios na genética da conservação podem ser traduzidos por desenvolvimento de projetos de restauração mais eficientes, seja do ponto de vista da biodiversidade como da dinâmica ecológica. Acredita-se que nos próximos anos observemos estratégias integradas de ecologia molecular com metodologias específicas para restauração de florestas tropicais.

Palavras-chave: restauração florestal, genética da conservação, ecologia molecular, genética de populações.

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INTRODUCTION

The new Brazilian forest code was approved by the national congress in 2011, when it had high repercussion and occupied considerable media time. The new legislation was sanctioned with several vetoes by the president and new discussion and votes in congress brought up a delicate subject. In this great debate about the Brazilian forests, we understood that policies should be reviewed, giving goals and duties to reach sustainability and preservation of our natural resources. Moreover, such decisions brought to the population the concern of how to deal with resources and which goals should be targeted. For many, the "Rio + 20" World Forum in 2012, brought a few concrete actions of forest conservation, but left profound environmental questions to second plan to many governments.

The country has a high biological richness (Giulietti et al., 2005; Vitule, 2012), which has been unsustainably exploited over the years, especially with the development of Brazilian agriculture. The legal reserves, water springs zones and other permanent preservation areas bring benefits to all sectors, including agribusiness. According to Galdolfi (2011), discussion of this topic is vast and complex, and legal reserves are a strategic and invaluable tool for the economic, social, scientific and technological development of Brazil.

The restoration of areas that have been degraded by human activity is essential for the sustainability of the environment and allows for the connection of forest patches. Thus, it becomes necessary to map priority areas for restoration and conservation, aiming to establish a policy that reconciles agroecology productivity and environment conservation (Rogalski et al, 2003; Joly et al, 2010).

With the increasing demand for recovery and management of these areas, it is essential that theoretical concepts about the composition, structure and functioning of tropical ecosystems are undertaken for the construction of appropriate technologies for these actions. Besides the concern for forest restoration, it is also important to recover the complex networks of inter and intraspecific interactions and to make possible the long-term conservation of habitats and organisms (Koskela et al., 2003).

The fusion of concepts and practices of population ecology and population genetics is essential to guide the actions to be undertaken in the field of biology conservation (Kageyama & Gandara, 1998). However, despite the broad theoretical basis for population genetics studies found in the literature (Allendorf & Luikart, 2006), the application to issues such as conservation and management of natural populations disturbed by anthropogenic factors is still recent (Lowe et al., 2005), especially in Brazil (Kageyama & Gandara, 2004). More incipient are the studies on restoration ecology to assist the definition of more efficient strategies for reforestation of degraded areas (Engel & Parrotta, 2001; Leopold et al., 2001), especially as regards the restoration of genetic diversity of tree populations (Rodrigues et al., 2009). The use of molecular markers in population genetics studies allowed the development of a new way of analyzing population patterns and relationships between individuals of the same species. The Molecular Ecology is showing up as a study area with several applications, among them, the conservation of species, ecosystems and forest restoration. The purpose of this article is to underline how Molecular Ecology can be applied to forest restoration and what is the return of these investments in the quality of forest restoration projects.

Molecular biology in the service of forest restoration

Molecular markers are one of the main tools in Molecular Ecology studies. They are landmarks in the chromosome, where it is possible to verify the genetic polymorphism at the DNA level (Grattapaglia & Ferreira, 1998). These markers are used to understand the population's genetic diversity and structure, and also to determine the reproductive system of these species, to test hypotheses of migration patterns, and to understand how the processes of gene flow and genetic drift are affected due to landscape fragmentation (Heywood & Iriondo, 2003; González-Martínez et al., 2006).

In modern projects of forest restoration it is essential to take into account the richness of species and their genetic diversity, considering the consequences of the level of genetic diversity located in the target area. Thus, population genetics is critical to the design and implementation of any restoration project. It is directly related to the population's ability to evolve in response to environmental changes and

to adapt to the current environment in which it is found (Falk et al., 2006).

Genetic effects resulting from habitat loss are important factors to be considered in the study of genetic diversity. Since the anthropic action on forest areas usually reduces the size and number of the populations, the effects of genetic drift over them become more pronounced. Genetic drift is the change in genetic composition of populations as a result of chance. Common consequences of habitat loss and isolation of forest fragments are reduced genetic variability (H_e), smaller effective population size (N_e), and the possible increase of inbreeding (F_{IS}) among loci (Hartl & Clark, 1997). These genetic effects can have serious consequences for plant populations, such as reduced reproductive success and ongoing population reduction (Nason & Hamrick, 1997).

Another important issue to be discussed is the influence of deforestation on the genetic structure of populations, i.e., how the populations of each species are grouped considering their genotypes. Knowledge of the population genetic structure is essential to conservationists so that they can make changes in magnitude and desired direction. The replacement of the original vegetation by an anthropogenic landscape, in most cases by pasture or crops for agriculture, negatively influences the ability of species dispersion and consequently gene flow (Nm) between populations. In this scenario, it is commonly observed the increase of genetic structure (Hamrick, 2004; Haag et al. 2010), usually calculated by the estimator Θ (Weir & Cockerham, 1984). From the viewpoint of metapopulation, reduced gene flow between demes increases the effect of stochastic events such as genetic drift, which may radically reduce the persistence time of species (Hanski, 1991). Forest restoration is an important tool to minimize the isolation of populations. If the restored areas work effectively as a stopping point for pollinators and seed dispersers, gene flow can be restored and the effects of genetic drift can be slowed, i.e., reducing the loss of genetic diversity (Young et al., 1996).

The current restoration model accepted by the scientific community has an emphasis on the recovery of ecological processes that lead to the development of plant communities (Brancalion et al., 2009). However, there is great concern about the diversity of species, but little attention is given to intraspecific diversity. Generally, seedlings introduced in reforestation areas have low genetic diversity, because they come from few seed matrices, which can generate the same negative consequences of fragmentation (Brancalion et al., 2009). Recent studies indicate that it is of vital importance to select source populations with high genetic diversity and collect a random sample of seeds, respecting the minimum number of trees (Kageyama & Gandara, 2000). For an effective size of at least 50 individuals in the restored population, it is suggested to collect seeds from at least 12 matrices (Brancalion et al., 2009)

Even if forest restoration has been done with care to maintain genetic diversity, it is also important to note if this diversity can be maintained in the long term, since there is a downward trend over the generations. Inbreeding and fine-scale structure, on the other hand, are the most immediate indicators of the impact of the reduction in population and the restriction of seed dispersal (Lowe 2005).

The reforestation of degraded areas is critical when considering the current state of fragmentation of native ecosystems in the country. Reed and Frankham (2003) found a significant correlation between genetic variation and the likelihood of long-term survival of a population and that adaptability is reduced in small populations due to genetic drift and inbreeding depression. Considering the case of the Atlantic Rain Forest, which was reduced approximately to 11.7% of the original area of the biome in fragment areas (Ribeiro et al., 2009), forest restoration based on studies of population genetics can be seen as another tool for conservation.

Next challenges

Since the 1980's it is understood that biodiversity loss has been caused by man, and currently most of the scientific community agrees that the main challenge of this century is to prevent this loss of diversity at different levels: genes, species and ecosystems (Rands et al., 2010). Understanding "what, where and how to save" has gained priority in conservation biology, especially in species that inhabit major threatened hotspots (Brandon et al., 2005; Scheffers et al., 2012). Therefore, the conservation of species relies heavily on the concept of endemism, as well as the number of existing species. These decisions are best viewed using biogeographic methods that aim to understand critically the patterns of spatial distribution of organisms and respond to how these patterns were formed (Carvalho, 2009).

The rich native biodiversity of the state of São Paulo, Brazil, is threatened by changes in vegetation cover and effects of habitat fragmentation (Tabarelli et al., 2005; Brancalion et al., 2009). The Virtual Institute of Biodiversity BIOTA-FAPESP is a research program that focuses on conservation of biomes and one of its missions is to identify priority areas for forest restoration, with the goal of connecting forest fragments of native vegetation and select areas to create new conservation units (Joly et al., 2010). Integrated into this program, our group develops a contribution to the project entitled "Conservation Biology of native Atlantic Rainforest with phytotherapic potential: A genetic approach to forest restoration." One objective of this research is to understand the main differences between the remaining areas and areas undergoing restoration under the genetic point of view of some tree species using molecular markers to evaluate these differences.

With the development of microsatellite markers for forest species in this project, diversity and population genetic structure may be assessed. Furthermore, the use of a large number of samples and the application of markers such as AFLP, SNP, among others, in order to obtain a larger number of markers will, in an innovative way, compare the genomics of populations from degraded areas and forest remnants. The current project is expected to evaluate a possible methodology for enrichment of genetic diversity in previously reforested areas and contribute strongly to the field of molecular ecology applied to forest restoration.

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DNA e Meio ambiente,
um vídeo ilustrativo de como a Genética pode ajudar na conservação da biodiversidade

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Estudo sobre a diversidade genética e algumas aplicações desses conhecimentos para a conservação de espécies fundamentam o vídeo "DNA e Meio Ambiente". O curta de quatro minutos de duração apresenta o assunto de modo acessível para estudantes do ensino médio e para um público leigo que busque informações a respeito de Genética e conservação de espécies. O vídeo pode ser exibido em locais sem estrutura de som pois não há narração, apenas ilustram-se as aplicações. É possível assisti-lo e obter uma cópia do mesmo, gratuitamente, em http://vimeo.com/69343714

O conteúdo da animação centra-se em alguns temas, como a dos impactos ambientais causados pelas atividades humanas afetam a diversidade biológica e a qualidade de vida no planeta. Os cientistas, por sua vez, são apresentados como agentes que, ao estudarem os seres vivos, podem ajudar a mitigar a perda de biodiversidade e que a Genética, juntamente com outras áreas de estudo, pode ser uma ferramenta útil para auxiliar na compreensão de tais questões. Ressalta ainda que todos os seres vivos possuem DNA e que sua utilização não é restrita aos testes de paternidade. Também são apresentados conceitos usados pela Genética da Conservação, como:

1) ambos os genitores contribuem para composição do DNA do filho (hereditariedade);

2) existe variação nas sequências de DNA entre indivíduos da mesma espécie e de espécies diferentes (variabilidade genética); e diferenças genéticas levam a variações nas características dos indivíduos (variabilidade fenotípica).

São ressaltados alguns conceitos que são apresentados de forma simplificada. Por exemplo, o termo "gene" é usado tanto para indicar que se trata de um trecho do DNA, assim como uma versão daquele trecho de DNA (alelo). Os conceitos podem ser trabalhados com maior ou menor profundidade e detalhamento, dependendo da necessidade didático-pedagógica.

Dois exemplos ilustram aplicações da Genética em estudos de conservação. O primeiro trata da importância da diversidade genética em uma espécie de ave. Os indivíduos podem apresentar bico pequeno, sendo capazes de se alimentar de sementes menores, ou bico grande e se alimentar de sementes maiores. Com a extinção de plantas de sementes pequenas, aves de bico pequeno também desaparecem, mas não ocorre a extinção total da espécie por existir diversidade genética para o tamanho de bico. Contudo, em um cenário em que não há diversidade genética para esta característica, a ausência do tipo de alimento específico ao tamanho do bico levaria tal espécie à extinção.

O segundo exemplo trata dos problemas associados a cruzamentos entre parentes (depressão endogâmica). A super exploração de bromélias de uma floresta leva a uma drástica redução populacional. As bromélias remanescentes na mata são aparentadas e podem ter problemas reprodutivos e as novas bromélias geradas a partir de cruzamento entre parentes podem ter problemas em sua formação e desenvolvimento.

O vídeo também mostra que existe uma relação entre diversidade genética e variação fenotípica e que, quanto maior a diversidade genética, menor a chance de extinção de espécies. Assim, a animação ressalta que com o conhecimento da diversidade genética, os cientistas podem identificar espécies ou áreas prioritárias para conservação.

"DNA e Meio Ambiente" – além das qualidades já apontadas, oferece outras boas características como material didático: o uso de linguagem técnica adequada para aulas introdutórias sobre genética ou sobre conservação. Também pode ser utilizado em aulas para graduação, como forma de instigar o interesse dos alunos para um aprofundamento de assuntos como Genética da Conservação, Genética de Populações, diversidade intrapopulacional, entre outros.

Paper in preparation

1. Zucchi MI, Sujii PS, Mori GM, et al. Restoring genetic diversity in a threatened ecosystem.

Restoring genetic diversity in a threatened ecosystem

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Running title: genetic diversity in restoration

Abstract

New international commitments foster large-scale restoration projects. The long-term ecological success of these emergent projects will rely on the genetic diversity of reintroduced or colonizing species, which is a limiting factor in highly-fragmented landscapes. Despite the paramount role of genetic diversity for species persistence, the effectiveness of genetic diversity recovery in restoration programs is poorly known. By assessing the genetic diversity of four model tree species in restored and conserved sites in the Atlantic Forest of Brazil, we found that restoration areas show similar levels of neutral genetic diversity and inbreeding to those observed in natural forest remnants. Based on these findings, we advocate the use of high levels of genetic diversity in restoration in order to support biodiversity conservation in human-modified landscapes. We demonstrate how ecological restoration can be a powerful tool for not only supporting the conservation of ecosystems and species, as well documented in the literature, but also genetic diversity – the basic constituent of biodiversity.

Introduction

Recent international commitments have paved the way for the implementation of large-scale ecological restoration programs in the upcoming decades (Latawiec et al. 2015). The success of such programs will rely on the increase of ecological integrity and long term sustainability of restoration (Suding et al. 2015). One of the key aspects underlying ecological integrity and sustainability is genetic diversity, which influences the chances of reintroduced or naturally colonizing populations persisting in restored sites without further human assistance (Mijangos et al. 2015). However, the role of genetic diversity in restoration processes represents a knowledge gap for the effective implementation of restoration programs.

Threatened ecosystems, where severe habitat loss and fragmentation increase the risk of extinction after habitat change, require special attention to genetic issues (Kuussaari et al. 2009). Following drastic reductions in population size and gene flow, some species may go extinct due to increased genetic load, i.e. accumulation of deleterious recessive alleles; reduced fecundity, and hindered adaptability as a result of genetic drift and inbreeding (Young et al. 1996, Aguilar et al. 2008). Consequently, protecting existing fragments, increasing habitat cover, and reconnecting

habitat patches through restoration interventions represent a major strategy for mitigating loss of biodiversity (Possingham et al. 2015). Threatened ecosystems are also dominated by second-growth remnants. In the case of tropical forests, over 70% of their global cover is constituted by naturally regenerated fragments (FAO 2010). Tree populations in second-growth tropical forests can initially have low levels of genetic diversity reflecting a strong founder effect, and shift towards genetically-rich populations in the mid- and long-term due to gene flow at the landscape level (Sezen et al. 2005, 2007). This gene flow between remaining and restored patches may be compromised in threatened ecosystems due to reduced habitat cover and severe fragmentation. This scenario enhances the need for restoration projects aimed at planting populations with higher levels of genetic diversity, in order to ensure some level of autonomous viability among the restored populations, until gene flow is restored. Consequently, the implementation of restoration projects that use an initial pool of individuals representing high levels of genetic diversity can help achieve both ecological sustainability for restored patches and provide a source of alleles and genes for remaining populations.

In spite of its strategic importance for conserving biodiversity in threatened ecosystems, ecological restoration has been mostly recognized and supported by society because of its role for improving ecosystem services (Palmer & Filoso 2009). For instance, 86% of restoration projects implemented in Colombia focused in watershed services (Murcia et al. 2015), a trend also observed in other restoration projects in Latin America (Brancalion et al. 2014). Fostered by society awareness, restoration policies have been focused in reestablishing ecosystem functions in degraded areas with importance for soil and water protection. Overall, 60% of studies included in a review about restoration success were implemented to comply with environmental laws with clear links with ecosystem services provisioning, like the Clean Water Act in USA (Ruiz-Jaen & Aide 2005). Payments for ecosystem services (PES) schemes reinforced this trend. A review about PES in the Brazilian Atlantic Forest indicated that only 5 out of 79 projects focused on biodiversity conservation, while carbon stocking and watershed protection were the main targets (Guedes & Seehusen 2011). While a growing body of empirical and scientific evidence has supported the role of restoration for recovery of ecosystem services, the same cannot be said about its ability to reestablish similar composition levels to reference ecosystems (Rey Benayas et al. 2009; Bullock et al. 2011; Suganuma & Durigan 2014). When genetic diversity is considered, the knowledge gap is

even bigger, which limits science-policy interface for the inclusion of genetic concerns as part of the strategic plan for the implementation of global restoration commitments in the coming decades.

In the Brazilian Atlantic Forest, one of the top five global biodiversity hotspots, the predominance of landscapes with less than 10% habitat cover illustrates the need to upscale restoration programs to safeguard biodiversity (Banks-Leite et al. 2014). Restoration projects aimed at high levels of species diversity have been implemented in the last two decades in this biome (Rodrigues et al. 2011), but with little attention to the genetic diversity of reintroduced species. The same is true for the restoration of other species-rich ecosystems worldwide, in which restoration practitioners are still struggling to address taxonomic and functional diversity, with concerns over genetic diversity remaining a relatively minor issue.

We assessed the genetic diversity of four tree species in old restoration plantations and conserved forest remnants. We tested the following hypotheses for four functionally different species to evaluate the potential for restoration projects to provide sources of alleles among fragments through gene flow: (i) there is substantial genetic differentiation among study populations due to the approach adopted by early restoration projects and the geographic distance between natural areas; (ii) restored populations have lower genetic diversity and higher inbreeding levels than populations from natural forest remnants.

Methods

Study sites

We studied four areas within the Brazilian Atlantic Forest, in the state of São Paulo: two areas undergoing restoration and two natural remnants (Figure 1). All fragments selected for this study lie in the seasonal semideciduous forest domain, within the Atlantic forest complex, with Cwa Köppen climate classification.

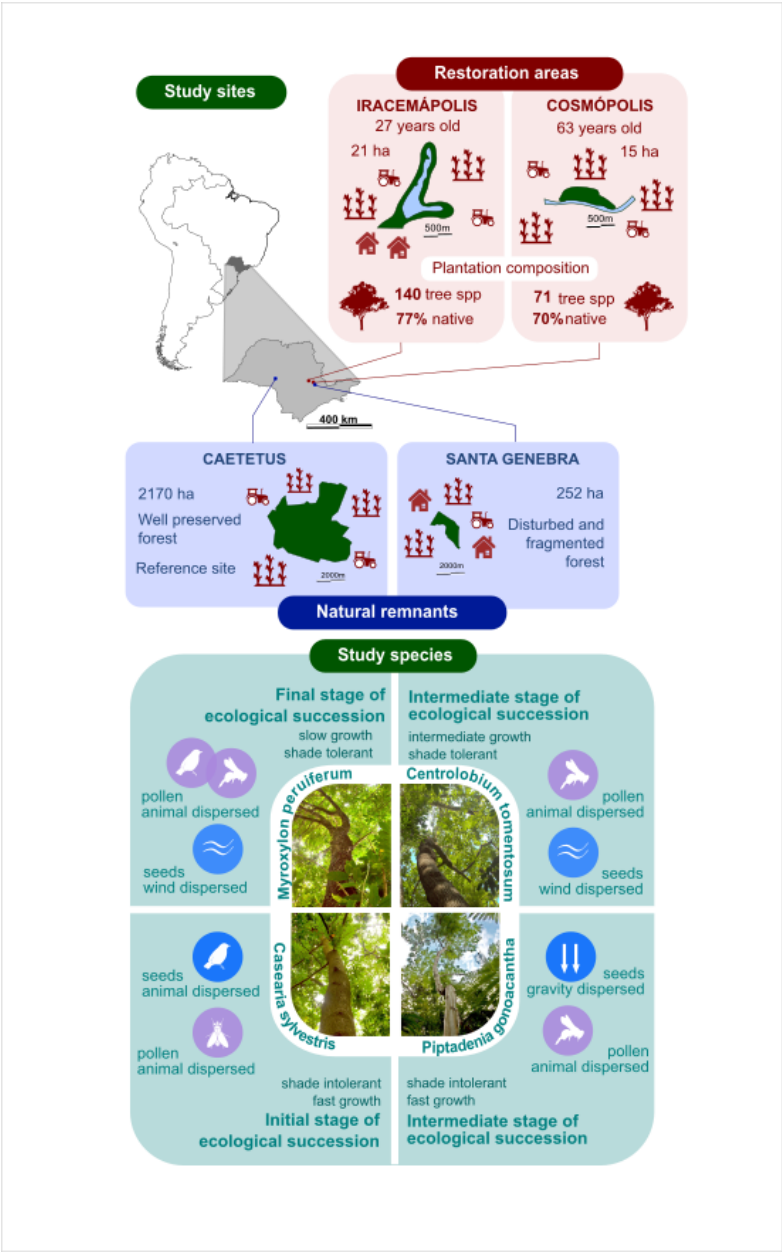

Figure 1. Description of studied sites and species in the Atlantic Forest region of São Paulo state, Brazil.

Both restoration sites were established in riparian buffers previously occupied by sugarcane plantations in the Iracemápolis (Rest.1) and Cosmópolis (Rest.2) municipalities. The restoration approach for these areas has been based on establishing high species-diversity (see details in Garcia et al. 2014), and the landscape matrix in which they occur is dominated by sugarcane plantations, which has very low native forest cover remaining (5.6% for Iracemápolis and 10.5% for Cosmópolis municipalities).

The natural remnants were selected to represent a large conserved ecosystem and a fragmented, disturbed forest patch, in order to contrast the genetic status of populations in conserved areas with those in remnants subject to strong fragmentation, which predominately comprise the Atlantic Forest region (Ribeiro et al. 2009). Caetetus Ecological Station (Cons.) served as the reference ecosystem, as it is a well-preserved and large forest patch (2170 ha), surrounded by agricultural areas and pastures (Durigan et al. 2000). The Municipal reserve of Santa Genebra Forest (Frag.) represented the disturbed forest and is the largest urban, semideciduous, seasonal forest fragment in São Paulo State (252 ha). In contrast to the Cons., Frag. has been compromised by human-mediated disturbances (Farah et al. 2014).

Study species

We studied the tree species *Casearia sylvestris* (Salicaceae), *Centrolobium tomentosum* (Fabaceae), *Myroxylum peruiferum* (Fabaceae) and *Piptadenia gonoachanta* (Fabaceae), which represent different ecological, pollination, and seed dispersal groups (Figure 1). They were also selected because there were a sufficient number of adult individuals in each site for genetic diversity analysis and spontaneously regenerating seedlings in the understory of plantations for further studies on gene flow. These species were initially planted in Rest.1 using nursery grown seedlings produced in the same farm where the plantation was implemented, and in Rest.2 using seedlings produced in the forest nurseries of the Botanical Institute of São Paulo and of the Department of Water and Electric Energy of São Paulo State. Unfortunately, there is no information regarding the number of populations and mother trees from which the seeds used to produce the seedlings were collected. However, such pioneer restoration projects were known to focus in taxonomic plant diversity, without concerns about genetic diversity (Rodrigues et al. 2009).

Sampling

We sampled a total of 468 adult individuals across the four species in all sites (with an average of 31.2 individuals, min = 14, max = 50, for each species and sampling locality). Whenever it was possible, we sampled adult trees present in the original planting lines of restoration sites in

order to better include planted individuals in our samples. We collected leaves or a disc of vascular cambium from each tree for DNA extraction.

Molecular markers and genotyping

We quantified the genetic diversity of the four selected species using previously developed microsatellite markers. Seven loci were genotyped for *C. tomentosum* (Sujii et al. 2015), eight for *M. peruiferum* (Schwarcz et al. 2014), eight for *P. gonoachanta* (Grando et al. 2015) and eight for *C. sylvestris* (Cavallari et al. 2008). Genetic markers were enriched using the Polymerase Chain Reaction (PCR) following the amplification conditions described in the aforementioned studies. We genotyped amplified fragments on a Li-Cor 4300 DNA Analyzer (Li-Cor Biosciences, Lincoln, NE, USA) using the 50-350bp IRDye700 and 800 (Li-Cor) ladder and identified alleles with the Saga v. 3.3 software (Li-Cor).

Genetic analyses

We examined genetic population structure using the multilocus clustering method implemented in STRUCTURE 2.3.3 (Pricthard et al. 2000) under an admixture model with correlated allele frequencies. We performed 50 independent Markov Chain Monte Carlo runs for the number of clusters (K) ranging from one to 10 with 1×106 iterations following a burn-in period of 5×105 iterations. The uppermost hierarchical level of genetic structure was identified using K inferred with the ad hoc ΔK statistic (Evanno et al. 2005), which best explained the genetic data. We also quantified population subdivision by estimating F_{ST} (proportion of the genetic variance between subpopulations relative to the total genetic variance) using the R package diveRsity (Keenan et al. 2013). Confidence intervals were obtained with 1,000 bootstrap replicates. We used expected (H_E) and observed heterozygosities (H_O) and allelic richness (Ar) to estimate the genetic diversity of each species in each sampling locality. We also estimated inbreeding coefficients (F_{IS}) for the populations within each sampling locality using the diveRsity (Keenan et al. 2013) and PopGenKit (Paquette 2012) R (R Core Team 2015) packages. Confidence intervals were obtained with 10,000 bootstrap replicates.

Results

The multilocus clustering analysis indicated that populations from natural remnant forests were genetically differentiated as expected due to the large distance between them (Figure 2). Populations from restoration areas were comprised of up to three distinct genetic clusters, some of which were similar to natural remnant populations. Although we observed genetic structure among populations within species, the exact pattern of differentiation was not the same for all species. Each sample site for *P. gonoacantha* represented a genetically unique population, with almost no admixture. Conversely, for *C. tomentosum*, we detected only two distinct genetic groups, with one being present in all populations and the other in one remnant and one restoration area. Populations of *M. peruiferum* and *C. sylverstris* were composed of either two or three genetic groups, with substantial admixture.

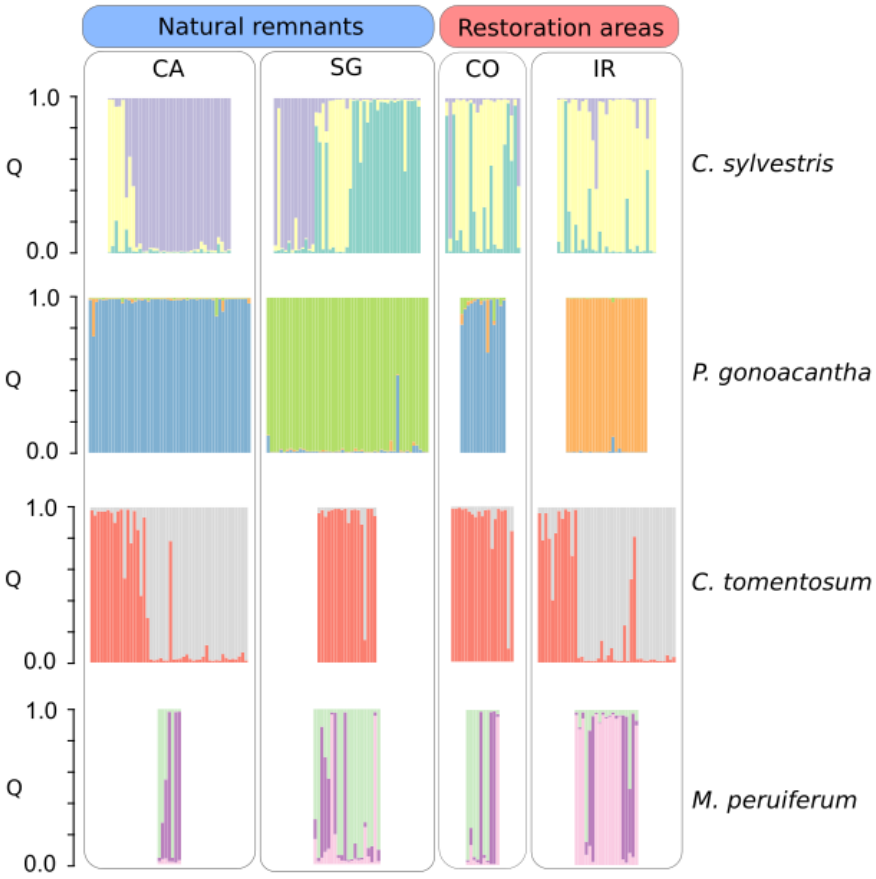

Figure 2. Genetic structure of all species and populations determined by the multilocus clustering method of STRUCTURE. Each column corresponds to a single individual and each colour represents a particular genetic assignment.

Overall, allelic richness was slightly lower or did not differ between restoration areas and natural remnants. Earlier successional species (*C. sylvestris* and *P. gonochanta*) showed the largest reduction in allelic richness between restored and natural populations compared to other species (Figure 3). In contrast, across all species the general pattern of estimated expected heterozygosity under Hardy-Weinberg Equilibrium and inbreeding coefficients for populations from restoration areas was not different from natural remnant populations (Figure 3).

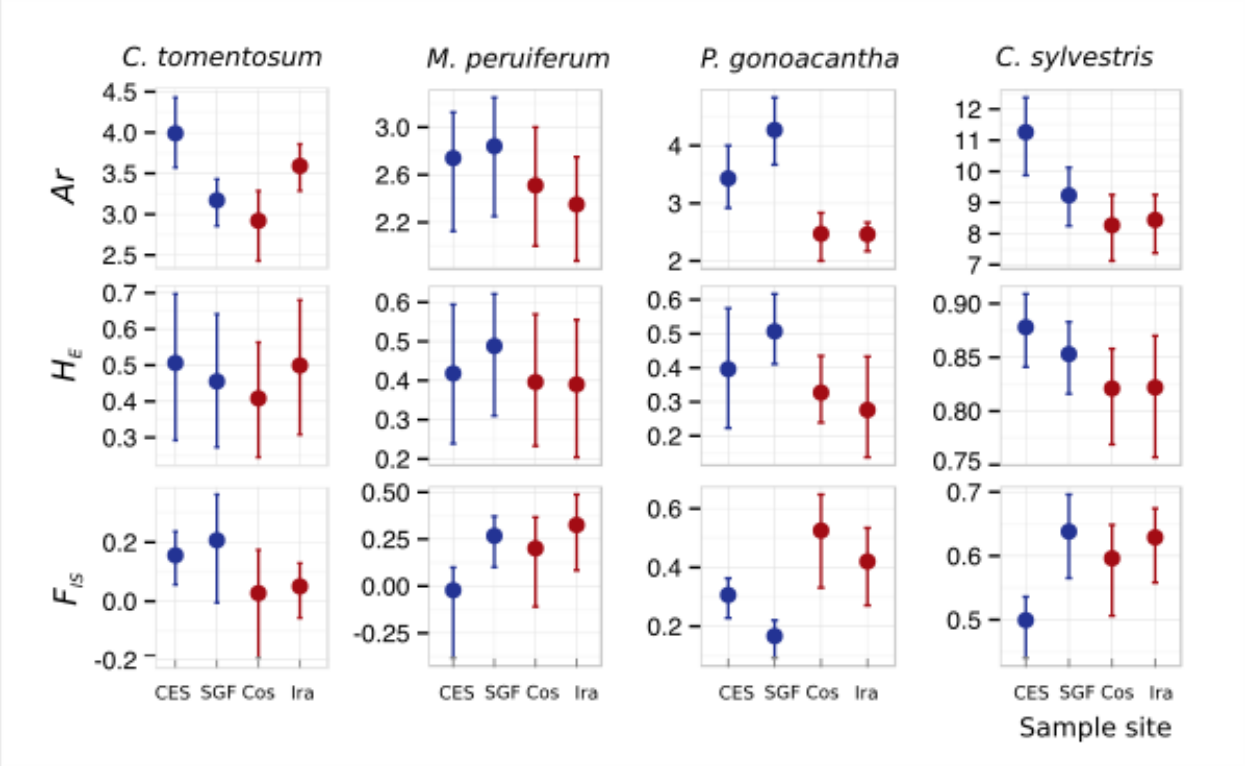

Figure 3. Estimates of genetic diversity (*Ar* - allelic richness; and H_E - expected heterozygosity under Hardy-Weinberg Equilibrium) and of inbreeding coefficients (F_{IS}) for each species. Blue: populations from natural remnants; Red: populations from restoration areas.

Discussion

Overall, we observed that populations in restoration sites had comparable levels of genetic diversity to those in natural forest remnants and were not exposed to higher levels of inbreeding depression. This pattern was consistent across all species, despite particularities detected for each taxa. Such favourable results indicate that it is fairly possible to reestablish high levels of genetic diversity when restoring degraded areas using seedling plantation and direct seeding – the most

commonly used restoration techniques described in the literature (Ruiz-Jaen & Aide 2005), and that it may support the persistence of reintroduced populations in the plant community by reducing the chances of genetic load even in the context of highly fragmented landscapes.

The persistence in restoration sites of reintroduced tree species with ecological importance for maintaining forest structure – like those included in this work – is one of the aspects to be considered to meet the call made by ecologists to policy makers to consider long term restoration sustainability as a planning principle (Suding et al. 2015). The healthy regeneration of such species in the plant community can help preventing biomass collapse even in the context of dispersal limitation, a common ecological barrier preventing the recolonization of large-seeded, late-successional tree species in tropical forest restoration projects (Reid et al. 2015). Ultimately, safeguarding the persistence of canopy tree species in restoration sites can help maintaining some of the functions they mediate, like carbon stocking and soil protection, with direct implications for ecosystem services. Establishing populations with high levels of genetic variation can also be a strategy to face global climate change.

Genetic variation in regions of the genome responsible for adaptation is required for populations to evolve in response to environmental changes (Allendorf et al. 2013). Although high genetic diversity in neutral regions of the genome does not guarantee adaptive potential, there is a significant correlation between neutral levels of genetic diversity and population fitness (Reed & Frankham 2003). Therefore, similar levels of genetic diversity in restored and natural remnant forests indicate that the fitness of restored populations may be robust to inbreeding. It is noteworthy that for this analysis we examined only adult individuals, which in the restoration areas represent the initially-planted saplings and are not the result of reproduction after restoration. These results are evidence that the seedlings used in restoration plantations were not more inbred than the ones from well preserved natural remnants. Although the long term maintenance of high levels of genetic diversity is uncertain, given the evident limitations imposed by severe fragmentation, it was clear that restoration was not depauperated in genetic diversity compared to reference sites, and this is a good beginning.

However, the higher reduction in allelic richness in early successional species sampled in restoration sites suggests that some species may be more susceptible to bottleneck effects and lose alleles at a faster rate than overall heterozygosity, which indicates that the challenge of restoring

genetic diversity can vary among species. The observed differences in genetic structuring across species may be due to idiosyncratic ecological and historical species characteristics such as demography, life history, evolutionary history and genomic architecture (Duminil et al. 2007), as well as different seed sources used for each species. Both allelic richness and genetic structuring can be manipulated in restoration projects due to two main strategies, namely the establishment of restoration projects in portions of landscape where gene flow is favoured and the selection of populations and mother trees to collect seeds. This first strategy can be operationalized through the selection of restoration sites based on landscape connectivity, using prioritization maps already available for the Atlantic Forest (Tambosi et al. 2014). The second can be achieved through a well-designed seed collection program to increase genetic diversity of seed lots, which can include selecting populations and mother trees and mixing seed lots obtaining from different sources to maximize genetic diversity (Brancalion et al. 2012). Therefore, managing genetic diversity is not only important but also viable in Atlantic Forest restoration and, potentially, elsewhere.

Establishing populations with high genetic diversity in restoration sites can be useful for supporting the persistence of restored populations, as well as for conserving populations in forest fragments, since these early restoration areas may be suitable nodes of forest connectivity in the landscape matrix and be a source of new alleles for previously isolated populations. Populations from restoration fragments can facilitate gene flow by acting as stepping-stones for genetic material bound for surrounding forest fragments, which in turn mitigates genetic drift in small restoration patches and in previously isolated tree populations (Figure 4). Since some restoration areas may be gene sources in fragmented landscapes, they could be used as key landscape components to support conservation genetics of species threatened by fragmentation. This reinforces the importance of maintaining and creating habitat patches for increasing landscape connectivity and consequent gene flow among remaining and reintroduced populations (Possingham et al. 2015), adding value to recent frameworks that propose prioritization of restoration sites to increase landscape connectivity (Rappaport et al. 2015). Such restoration patches could also serve as germplasm conservation sites to safeguard genetic diversity of vulnerable species, which might be particularly relevant in drastically transformed environments (Breed et al. 2012) such as the Atlantic Forest.

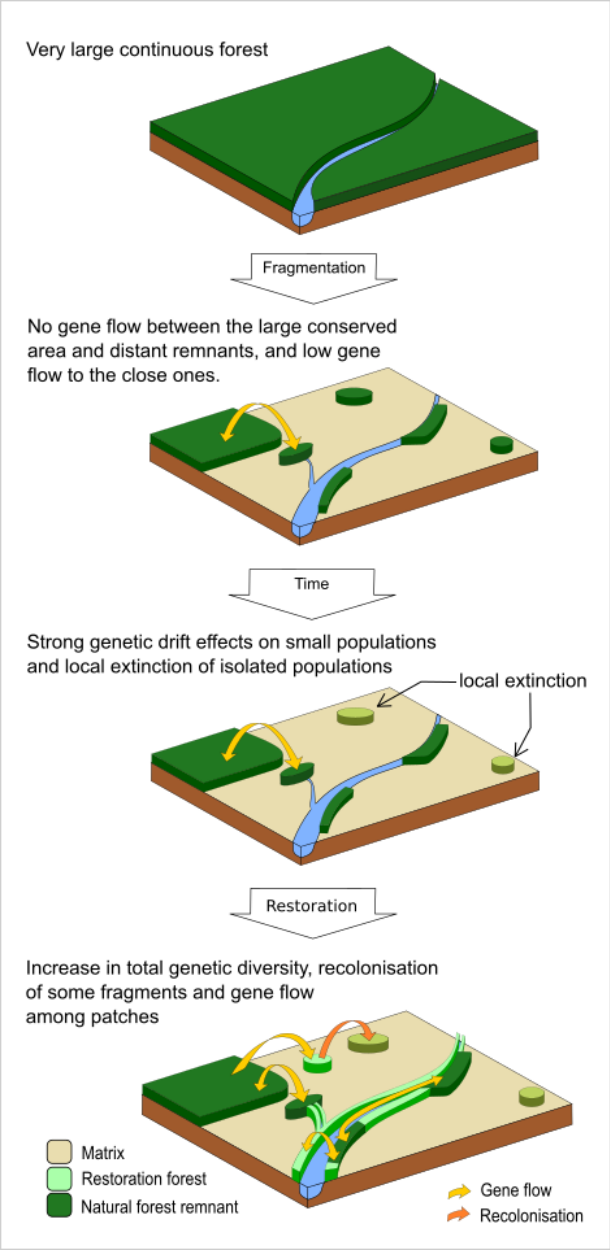

Figure 4. Expected effects of restoring small forest fragments with high genetic diversity.

Conservation genetics research should go beyond describing the ongoing trend of fragmentation-driven genetic impoverishment, and explore the new avenues offered by the emergent field of restoration genetics. Ecological restoration can be a powerful tool for not only supporting the conservation of ecosystems and species, as is well documented in the scientific literature, but also genes – the basic constituents of biodiversity.

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DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha , intitulada "Diversidade, estrutura genética e sistema reprodutivo em *Centrolobium tomentosum* Guillem ex. Bentham (Fabaceae) visando subsidiar enriquecimento genético em áreas de restauração florestal", desenvolvida no Programa de Pós-Graduação em Genética e Biologia Molecular do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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