GENETIC DIFFERENTIATION AND TEMPORAL ASPECTS OF THE FINE-SCALE GENETIC STRUCTURE IN FRAGMENTS-VEGETATION CORRIDORS: INFERENCES FROM A DIOECIOUS-DOMINANT NEOTROPICAL TREE

FÁBIO DE ALMEIDA VIEIRA

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do programa de Pós-graduação em Engenharia Florestal, área de concentração Ciências Florestais, para a obtenção do título de "Doutor".

Orientadora
Profa. Dra. Dulcinéia de Carvalho

LAVRAS
MINAS GERAIS – BRASIL
2009

Ficha Catalográfica Preparada pela Divisão de Processos Técnicos da Biblioteca Central da UFLA

Vieira, Fábio de Almeida.
Genetic differentiation and temporal aspects of the fine-scale genetic structure in fragments-vegetation corridors: inferences from a dioecious-dominant neotropical tree / Fábio de Almeida Vieira. – Lavras : UFLA, 2009.
96 p. : il.

Tese (Doutorado) – Universidade Federal de Lavras, 2009.
Orientador: Dulcinéia de Carvalho.
Bibliografia.

1. Allozymes. 2. Conservation genetics. 3. Reproductive biology. 4. Landscape structure 5. *Protium spruceanum* I. Universidade Federal de Lavras. II. Título.

CDD – 634.97324

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APROVADA em 3 de março de 2009.

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LAVRAS
MINAS GERAIS – BRASIL

AGRADECIMENTOS

A Deus, pela conquista. Aos meus pais e irmãos, pelo carinho, atenção e apoio durante a minha trajetória acadêmica. Ao sobrinho Gabriel, xodó da família, que nasceu junto com o meu Doutorado!

À Capes, pela concessão da bolsa de estudos durante todo o período do mestrado e doutorado (cinco anos). À Fapemig, pelo suporte financeiro deste trabalho (CRA 1770/05). Ao Programa de Pós-Graduação em Engenharia Florestal/UFLA. Em especial, agradeço à secretária Roseane. Aos professores do Departamento de Ciências Florestais/UFLA, pela grata convivência e ajuda em algum momento.

À orientadora professora Dra. Dulcinéia de Carvalho, pela oportunidade e confiança nestes cinco anos de convivência.

Aos professores Dr. Alexandre Magno Sebbenn, Dr. Flávio Nunes Ramos, Dra. Marines Marli Gniech Karasawa e Dra. Rosângela Alves Tristão Borém, pela participação na defesa da tese e sugestões. Ao professor Dr. Eduardo van den Berg, por ter acompanhado este trabalho, com sugestões e questionamentos. Aos professores da análise prévia dos artigos pelas contribuições para melhorar o trabalho (Dra. Cecile F.E. Bacles, Dr. Christian Lexer, Dr. Dion Devey, Dr. Eduardo L. Borba, Dr. Geoff Carr e Dr. Henk W.M. Hilhorst).

Ao Dr. Rubens Manoel dos Santos, pela amizade, competência profissional e sociedade (também financeira) em excelentes expedições, extremamente importantes ao estímulo. À Dra. Vivette Appolinário, pela grande amizade, companhia e colaboração nos estudos de fenologia e biologia reprodutiva, pela paz e paciência transmitida. À Dra. Gislene C. de Castro, pela amizade e informações demográficas da espécie *Protium spruceanum*.

À professora Dra. Maria Bernadete Lovato, MSc Renan M.L. Novaes e amigos do Laboratório de Genética de Populações, Evolução e Conservação da Biodiversidade/UFMG, pelo amparo e orientação nos estudos de filogeografia.

Aos amigos de curso e do Laboratório de Conservação Genética de Espécies Arbóreas/UFLA, Afrânio, Alisson, Cristiane, Elias, Emílio, Daniele, Daniel Leão, Daniel Pifano, Eduardo, Evânia, Flávio, Gabriela, Gleyce, Hugo, Joema, Maestro, Mirian, Olívia, Pablo, Renato, Ricardo, Rogério, Rute, Sue Ellen, Tati, Totonho, Vinícius, que bom, foram tantos... Aos amigos Hisaías e Murilo, pelos bons momentos que compartilhamos ao longo dos últimos anos.

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APRESENTAÇÃO

Os estudos que indicaram a relevância do sistema fragmentos-corredores de vegetação iniciaram-se em 2002, quando o trabalho desenvolvido por Castro (2004) destacou a importância dos corredores de valo de divisa para a conservação da flora local, em termos estruturais e de diversidade florística. A partir de 2004, a equipe do Laboratório de Conservação Genética de Espécies Arbóreas iniciou-se os trabalhos na área de genética de populações, fenologia reprodutiva e padrão espacial de espécies arbóreas na referida paisagem (Vieira, 2005; Brandão, 2008; Vieira & Carvalho, 2008; Vieira et al., 2008).

A presente tese organiza as informações obtidas para a espécie *Protium spruceanum* (Burseraceae) e está estruturada em quatro artigos, conforme a opção de formatação de dissertações e teses do Programa de Pós-Graduação em Engenharia Florestal. O primeiro artigo, publicado no periódico *Biodiversity and Consertavion*, apresenta a estrutura genética da espécie e as implicações para a conservação das populações naturais no sistema fragmentos-corredores. O segundo artigo, enviado para a revista *Acta Botanica Brasilica*, discute a diferenciação genética da espécie entre os fragmentos e corredores de vegetação. O terceiro artigo, preparado para submissão a *Revista Brasileira de Botânica*, investiga a biologia reprodutiva de *P. spruceanum* e as possíveis implicações ecológicas e genéticas. Finalmente, o quarto artigo, preparado para o envio ao periódico *Annals of Botany*, analisa os padrões espaciais demográficos e genéticos entre estágios de vida de *P. spruceanum*. Com este trabalho, espera-se melhor elucidar as possíveis causas das variações observadas e indicar os caminhos para novas pesquisas em conservação genética no sistema fragmentos-corredores de vegetação da região.

Lavras, 03 de março de 2009.

Fábio de Almeida Vieira

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VIEIRA, F. A.; BRANDÃO, M. M.; ARRIEL, D. A. A.; CARVALHO, D. Razão sexual e padrão espacial de *Lithraea molleoides* (Vell.) Engler (Anacardiaceae) em ambientes de fragmentos e corredores de vegetação em Lavras, MG. In: CONGRESSO NACIONAL DE BOTÂNICA, 59., 2008, Natal, RN. **Resumos**... Natal, 2008. CD-ROM.

VIEIRA, F. A.; CARVALHO, D. Genetic structure of an insect-pollinated and bird-dispersed tropical tree in vegetation fragments and corridors: implications for conservation. **Biodiversity and Conservation**, Netherlands, v. 17, p. 2305-2321, 2008.

RESUMO

VIEIRA, Fábio de Almeida. **Diferenciação genética e aspectos temporais da estrutura genética em escala fina em fragmentos e corredores de vegetação: inferências para uma espécie arbórea Neotropical dioica e dominante**. 2009. 96p. Tese (Doutorado em Engenharia Florestal) – Universidade Federal de Lavras, Lavras, MG. [1]

Foram estudados os padrões da diferenciação genética, da biologia reprodutiva e da estrutura genética e demográfica em escala espacial fina em uma paisagem conectada com interessante história de conversão humana do hábitat, que se iniciou há dois séculos, durante o período colonial. Nessa paisagem, *Protium spruceanum* é uma arbórea nativa abundante, com floração massiva, polinizada por insetos e dispersa por pássaros. Foram utilizados locos aloenzimáticos, dados reprodutivos e análises do padrão espacial para inferir sobre os processos que determinam a distribuição da diversidade genética em nível de paisagem e em escala espacial fina, ao longo de diferentes classes demográficas. Os resultados indicaram alta diversidade genética nos fragmentos e corredores, correlacionada positivamente com a densidade de plantas. Não foram observadas evidências de endogamia dentro dos fragmentos e corredores. Entretanto, evidências de gargalos populacionais recentes foram detectadas nos fragmentos, provavelmente decorrentes da fragmentação florestal. A diferenciação genética foi baixa e nenhum padrão de isolamento pela distância foi observado. Essa baixa diferenciação é, provavelmente, associada à extensa habilidade de *P. spruceanum* com relação ao fluxo gênico. As pequenas flores são funcionalmente unissexuais e dispostas em densas inflorescências. As flores estaminadas produzem grande quantidade de pólens viáveis e relativamente de néctar. Flores pistiladas oferecem apenas néctar para os polinizadores efetivos e oportunistas (*Apis mellifera* e *Trigona* sp.). Observou-se, em geral, menor diferenciação genética entre fragmentos e corredores vizinhos, indicando possível fluxo gênico por sementes e pólen. Em escala espacial fina, observou-se uma ligação entre a estrutura espacial genética e demográfica em pequenas distâncias (<10 m), provavelmente consequência da restrita dispersão de sementes. Foi observado decréscimo da magnitude do padrão espacial e da estrutura genética em escala fina com o avanço das classes diamétricas. Foram discutidas as implicações ecológicas dos resultados e os argumentos para explicar a alta diversidade genética da espécie na paisagem local. Assim, conclui-se que os corredores de floresta secundária podem ser uma alternativa na conexão genética de fragmentos isolados. Os resultados sugerem que as estratégias de manejo da paisagem devem considerar a proteção dos corredores de vegetação. Além disso, o estudo mostra que a diversidade genética pode ser mantida devido ao recrutamento natural pós-fragmentação, importante aspecto para a conservação *in situ*.

[1] Comitê Orientador: Dulcinéia de Carvalho - UFLA (Orientadora).

ABSTRACT

VIEIRA, Fábio de Almeida. **Genetic differentiation and temporal aspects of the fine-scale genetic structure in fragments-vegetation corridors: inferences from a dioecious-dominant Neotropical tree.** 2009. 96p. Thesis (Doctorate in Forest Engineering) – Federal University of Lavras, Lavras, MG. [2]

Patterns of genetic differentiation, reproductive biology and demographic fine-scale genetic structure (FSGS) were studied in a connected landscape with an interesting history of human habitat conversion that began two centuries ago, at Brazilian colonization period. In this landscape, *Protium spruceanum* is an abundant native, mass-flowering/insect-pollinated and bird-dispersed tree. We used allozyme loci, reproductive data and spatial pattern analysis to infer processes shaping the distribution of genetic diversity at landscape-level and fine-scale across different demographic classes. The results indicated high gene diversity in the fragments and corridors, positively correlated with plant density. We did not find evidence of selfing in the fragments or corridors. However, evidence of recent bottleneck was detected in the fragments. Genetic differentiation was low and no pattern of isolation by distance was found over a small geographical scale (< 2 km). This low differentiation is likely to be associated with the extensive gene dispersal ability of *P. spruceanum*. The small flowers are functionally unisexual and organized in dense inflorescences. Staminate flowers supplied a large quantity of viable pollen and relatively abundant nectar. Pistillate flowers produced only nectar to effective and opportunistic pollinators (*Apis mellifera* and *Trigona* sp.). All fragments generally have low historical genetic differentiation with corridors which they are connected, indicating possible gene flow via seeds and pollen. At fine-scale, there was a strong link between demographic and genetic spatial structures at small distances (less than 10 m) probably due to restricted seed dispersal. The magnitude of spatial pattern and FSGS decreased from smal- to large-diameter classes. Ecological implications of these findings and alternative arguments to explain the genetic diversity at regional landscape and FSGS are discussed. We conclude that corridors of second-growth forests would be an important alternative in the genetic connection of isolated forest fragments. Thus, our results suggest that landscape management strategies should therefore consider the protection of extant ones. Moreover, this study shows how genetic diversity can be maintained due to natural plant recruitment post-fragmentation, a positive aspect for *in situ* conservation.

[2] Advising Committee: Dulcinéia de Carvalho - UFLA (Advisor).

ARTIGO 1

Genetic structure of an insect-pollinated and bird-dispersed tropical tree in vegetation fragments and corridors: implications for conservation

(Publicado no periódico *Biodiversity and Conservation*, Netherlands, v. 17, p. 2305-2321, 2008)

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Abstract In the vegetation corridors that connect small remnants of undisturbed primary forest in the Lavras landscape (Brazil), *Protium spruceanum* is a representative of a mass-flowering insect-pollinated and bird-dispersed tree. Allozyme variation was quantified from five forest remnants (N = 150) from secondary vegetation corridors linking them (N = 80) to generate information for genetic conservation. The species adhered to HW equilibrium in all fragments in most of the loci. The results indicated high gene diversity in the fragments ($\hat{H}_e$ = 0.381 to 0.507) and corridors ($\hat{H}_e$ = 0.336 to 0.470), positively correlated with the plant density (r = 0.742, R^2 = 0.551, d.f. = 4). We did not find evidence of inbreeding within fragments ($\hat{f}$ = -0.188, $P < 0.05$) nor overall ($\hat{F}$ = -0.101, $P < 0.05$). The genetic differentiation among remnants was low ($\hat{\theta}_p$ = 2.8%). Evidence of recent bottlenecks by anthropogenic disturbance was detected in fragments ($P < 0.05$, Wilcoxon sign-rank test). The minimal viable population was estimated for conservation *in situ,* indicating fragments with possibilities of maintaining genetic equilibrium diversity in the short term (except F3) and in the long term (only F5). The $\hat{N}_e/N$ ratios was also calculated to contribute to vegetation enrichment, area recovery or creation of new vegetation corridors. We found high levels of gene diversity in the vegetation corridors, genetic identity with the fragments and absence of inbreeding. Thus, our results suggest that landscape management strategies should therefore consider both the creation of new vegetation corridors and the protection of extant ones.

Key words: Allozymes, Conservation genetics, Habitat fragmentation, Landscape structure, Minimum viable populations, *Protium spruceanum*, Vegetation corridors

1 Introduction

Tropical forests present very diverse terrestrial ecosystems, but much of this diversity is threatened by habitat destruction and extensive fragmentation of natural populations (Myers 1986). Forest loss and fragmentation alter the composition, structure and connectivity of the landscape (Taylor et al. 1993). Studies have shown that forest fragmentation negatively affects plant reproductive success by reducing pollinator activity (Aizen and Feinsinger 1994; Quesada et al. 2004), pollen deposition (Cunningham 2000; Cascante et al. 2002), fruit and seed set (Ghazoul et al. 1998; Fuchs et al. 2003; Quesada et al. 2004) and the regeneration of many species (Cascante et al. 2002; Benitez-Malvido & Martínez Ramos, 2003). On the other hand, some studies have shown a positive effect of forest fragmentation on pollen flow (White et al. 2002; Dick et al. 2003), no effect on fruit and seed production (Cascante et al. 2002), and an effect on long seed distance dispersal (Bacles et al. 2006). The impacts of forest fragmentation on genetic resources have also been widely discussed (Lowe et al. 2005). These results indicated that tropical tree species have different responses to forest fragmentation and suggest that, for some species, trees isolated in fragmented landscapes may have considerable importance in conservation (Lowe et al. 2005).

Creation or preservation of landscape structures, such as vegetation corridors, has been indicated to minimize the effects of habitat fragmentation. The proposal that corridors may increase organism migration among the isolated remnants has been much discussed in conservation biology, and is always mentioned in management plans as an important factor for biological conservation in fragmented landscapes (Simberloff et al. 1992). However, there are few scientific studies that justify the use of corridors for conservation (Rosenberg et al. 1997). Some have shown that, in some cases, corridors may increase the migration rates of species among the isolated remnants (Andreassen et al. 1998; Haddad 1999; Mech and Hallet 2001). However, most deal only with corridor importance for fauna conservation and little is known about corridor importance for plant species (but see Johansson et al. 1996; Kirchner et al. 2003). Also, previous work on this topic was not focused on population genetic processes, especially in the Neotropical region.

The conservation of the habitat biodiversity within the fragments depends on the particular capacity of the species to survive genetic bottlenecks and stochastic events. Thus genetic conservation studies play an important role as they interpret the survival mechanisms of populations threatened by ecosystem fragmentation (Templeton et al. 1990; Young and Clarke 2000). For this, it is necessary to access the genetic diversity and structure in the natural populations and test for associations with various characteristics of the environment or the species. The genetic diversity levels may depend on factors

including population size, environmental heterogeneity, plant density and landscape structure (Ellstram and Elam 1993; Franceschinelli and Bawa 2000; Manel et al. 2003). For example, rare species with large populations can show high levels of genetic diversity (Ellstram and Elam 1993), but can become more susceptible to founder effects and selective pressures because of local selection associated with differences in the microhabitat (Loveless and Hamrick 1984). Combining landscape ecology and population genetics, through the spatial mapping of allele frequencies from one or more populations and, subsequently the correlation of such patterns with the current landscape, may provide greater insight into how landscape characteristics structure populations (Manel et al. 2003).

The forest remnants in the state of Minas Gerais, Southeastern Brazil, are characterized by a hilly relief covered by vegetation mosaics formed by the contact between Atlantic seasonal forests, *cerrado* (woody savanna) and montane grasslands (Pereira et al. 2006). Despite the forest characteristics, such as species richness and diversity, species composition, tree density, biomass and, consequently, international importance of ecosystems for conservation of biodiversity, most of the remnants of these forests are in either small fragments or larger areas sheltered on steep mountain slopes (Oliveira Filho and Fontes 2000). These plant formations were seriously plundered since European occupation goes back to colonial times (two centuries a go). The vegetation corridors (~ 6 m-wide) that connect small remnants of undisturbed primary forest are the result of colonization by native tree species in the ditches constructed by slaves to divide rural properties in the colonial period. These corridors may be considered essential for conservation, because of their considerable floristic diversity and the occurrence of species unique to this environment (Castro 2004).

The ecological and genetic consequences of anthropogenic fragmentation are being investigated in a range of species, with different life history characteristics, to inform conservation management of remnant native vegetation in the area (Bacles et al. 2006, Jump and Peñuelas 2006). *Protium spruceanum* has been selected for this research as representative of a mass-flowering insect-pollinated and bird-dispersed tropical tree that occur in the fragments and corridor systems studied. The species is widely distributed and normally occurs in relatively large populations in association with water courses. Where fragments and vegetation corridor areas coexist, the historic fragmentation of these plant formations provides the opportunity to assess the effects of chronic habitat fragmentation, the influence of vegetation corridors and to test theoretical predictions. Specifically, the objective of the present study was to characterize the genetic structure of *P. spruceanum*, quantify the genetic variability in the populations in the fragmented and corridor landscape

and estimate the effective size of the populations, aiming to provide genetic information for *in situ* conservation strategies.

2 Materials and methods

Study species

Protium spruceanum (Burseraceae) is a widely distributed Neotropical tree species, found in the Amazon and the Atlantic rain forests and on the savannas inside riverbank woods (Oliveira-Filho and Ratter 1995). The species produces a fragrant resin used in popular medicine, the varnish industry, to caulk boats and as a perfume (Machado et al. 2003). In the study region, the species is a shade tolerant climax species and prefers wet environments (Castro 2004). *P. spruceanum* produces flowers with greater intensity between September and October (F.A.V, unpublished data). The flowers are functionally unisexual in dense inflorescences and the individuals are dioicous. Staminate flowers supply a large quantity of viable pollen (> 88%, stained by acetic carmine) coinciding with the reproductive phase of the stigma of the pistillate flowers (peroxidase activity method) that manifests itself immediately after flowering and lasts throughout the day (F.A.V, unpublished data). Staminate and pistillate flowers produce relatively abundant nectar (~ 4 μL), with an average of 30% concentration in sucrose equivalents. The effective pollinators are *Apis mellifera* and *Trigona sp* (Hymenoptera, Apidae) (F.A.V, unpublished data). Fructification occurs in September. Fruits are sub-globoid, reddish berries, containing one to two seeds surrounded by a sweet aril that are dispersed by birds.

Study site and populations

The fragment-corridor system studied is located in the region of Lavras, South of Minas Gerais state, in Brazil (Figure 1). The following can be observed in the current landscape: a) few and small forest remnants of primary forest, b) a matrix consisting of coffee plantations and planted pastures (*Brachiaria* spp.) for livestock rearing and c) vegetation corridors of secondary forest. The fragments studied showed evidence of localized impacts, caused by cattle entering the forest, damaging regeneration in several stretches. Fragments and vegetation corridors are pretty similar in terms of floristic composition, but corridors are denser, have larger basal area and trees are concentrated in the upper diameter classes and lower height classes.

Five interlinking fragments and a vegetation corridor were analyzed (Figure 1 and Table 1). The fragmentation and isolation of these populations has occurred for over two centuries and at a particularly rapid pace in the colonial

period. Thus, the estimated age of the trees sampled is of 2 – 4 generations before present, assuming a generation time of 50 – 100 years. All the fragments contained a water course in their interior. *P. spruceanum* is the most abundant tree species in the fragments. The presence of *P. spruceanum* in fragments F2 and F4 coincides with the presence of water courses. In fragments F1 and F5 the species occurs in a large proportion of the fragment, which may be determined by the almost permanently flooded soil. Fragment F3 is the result of a recent colonization in a small canyon. *P. spruceanum* is one of the most abundant in the vegetation corridors.

We sampled adult individuals in the fragments randomly, with diameter at breast height > 20 cm and ~ 16 m-height. The sampling in each corridor axis was along the length of the each corridor axis (Table 1). All sampled leaf material was kept on ice until transportation to the laboratory, where it was stored at -80 ºC until enzyme extraction.

Electrophoresis procedures

For enzyme extraction 200 mg leaf tissue were used per 1 mL of the extraction solution (buffer nº 1 of Alfenas et al. 1998). The extracts obtained were centrifuged at 12,000 rpm at 4°C for 10 minutes. After centrifuging, 20 µL of the supernatant was placed in the gel wells to proceed to the electrophoretic runs. Discontinuous vertical electrophoresis in a polyacrylamide gel was used, with the concentration and separation gels at 4% and 10.0%, respectively, and run at 4 °C for 3 h (constant current of 80 mA, and voltage of 300 V) (Alfenas et al. 1998). Eight enzyme systems were used: alcohol dehydrogenase (*Adh*, E.C.1.1.1.1), glucose dehydrogenase (*Gdh*, E.C.1.1.1.47), □-galactose dehydrogenase (*Gldh*, E.C.1.1.1.48), glutamate dehydrogenase (*Gtdh*, E.C.1.4.1.3), malate dehydrogenase (*Mdh*, E.C.1.1.1.37), peroxidase (*Per*, E.C.1.11.1.7), sorbitol dehydrogenase (*Sdh*, E.C.1.1.1.14) and shikimate dehydrogenase (*Skdh*, E.C.1.1.1.25). The *Mdh* and *Per* enzyme patterns showed two polymorphic loci; so we used ten polymorphic loci to genotype the individuals. Staining protocols and the genetic basis of allozyme banding patterns were inferred from segregation patterns with reference to typical subunit structure and conceptual methods described in Alfenas et al. (1998).

Data analyses

Genetic diversity

The following genetic diversity parameters were estimated using the program BIOSYS 2 (Swofford and Selander 1997): proportion of polymorphic loci ($\hat{P}$), mean number of alleles per locus ($\hat{A}$), observed heterozygosity ($\hat{H}_o$) and Nei's gene diversity ($\hat{H}_e$) (Berg and Hamrick 1997). A locus was considered

polymorphic if the frequency of the most common allele does not exceed 0.95 (Nei 1987). The percentage of loci in genetic equity (low variation amplitude) was calculated, with allele frequencies among 0.350 and 0.650 (Frankel et al. 1995). After analyzing the homogeneity of the variances and normality of distribution by the Kolmogorov-Smirnov test, the gene diversity ($\hat{H}_e$) was submitted to analysis of variance by the *F* test (ANOVA) and the means of the fragments and corridors were compared. We adjusted the significance level for multiple pairwise comparisons using a sequential Bonferroni correction (α = 0.005) (Zar 1999). In addition, the Pearson correlation coefficient (*r*) was calculated between $\hat{H}_e$ and plant density.

F-*statistics*

The parameters estimated were the coancestry coefficient among individuals within populations ($\hat{\theta}_p$), the over all fixation index ($\hat{F}$) and within population fixation index ($\hat{f}$) (Weir and Cockerham 1984). Confidence intervals at 95% probability were established for each population using the bootstrap procedure with 10,000 repetitions (Weir 1996). The analyses of variance and the bootstraps were carried using the GDA (Lewis and Zaykin 2000) and FSTAT 2.9.3.2 programs (Goudet 2002). The Wright fixation index ($\hat{f}$) was obtained using the GENETIX 4.05.2 program (Belkhir et al. 2004). The genotypic frequency deviations obtained compared to the expected frequencies by the Hardy Weinberg (HW) proportions were estimated and tested using the Fisher exact test and the BIOSYS 2 program.

UPGMA

As a measure of genetic identity between pairs of fragments, the genetic identity of Nei (1978) was used and then dendrograms were constructed using the UPGMA (Unweighted pair group method arithmetic average) method. The clustering obtained was assessed by the cophenetic correlation coefficient by comparing the identity similarity matrix with cophenetic similarity. The multivariate genetic identity analysis (UPGMA) and the cophenetic correlation were obtained using the NTSYS 1.5 package (Rohlf 1989).

Minimum viable populations

The effective population size ($\hat{N}_e$) was estimated using the components of variance method (Crow and Kimura 1970; Vencovsky 1997). The minimum viable populations (*PMV*) calculated corresponds to the number of necessary individuals for the maintenance of the genetic equilibrium diversity of the population. The difference ($\hat{D}$) between the population size estimated for each fragment ($\hat{N}$) and the *PMV* was calculated ($\hat{D} = \hat{N} - PMV$), that is, $\hat{D} = DA.A$

– ($\hat{N}_{e(\ reference\ )}$ /($\hat{N}_e$ /*N*)), where *DA* is the density of the species (ind.ha^{-1}), *A* is the area of fragment (ha), $\hat{N}_{e(\ reference\ )}$ is the effective size of reference (150 or 1500, for conservation in the short or long term, respectively) and *N* is the sample size of each fragment. The effective size of reference adopted was according to Nunney and Campbell (1993).

Departure from random mating

We used the BOTTLENECK 1.2.02 program (Cornuet and Luikart 1996) to test for significant recent decreases in $\hat{N}_e$. These tests are based on the principle that populations that have gone through a severe and recent genetic bottleneck show a faster reduction in the number of alleles than in the $\hat{H}_e$. Luikart et al. (1998) demonstrated that populations that have undergone a recent bottleneck show a transient excess of heterozygotes. This means that $\hat{H}_e$ becomes greater than the expected heterozygosity on balance between mutation and drift ($\hat{H}_{eq}$), because this is calculated from the number of alleles (Cornuet and Luikart 1996). Consequently, in a population that suffered a recent bottleneck, $\hat{H}_e$ will be higher than $\hat{H}_{eq}$ ($\hat{H}_e > \hat{H}_{eq}$). The analysis was carried out only on the loci in H–W equilibrium (as suggested by Lee et al. 2002), and all enzyme loci are assumed to fit an infinite allele model of mutation (IAM) (Kimura and Crow 1964). The significance was assessed using the Wilcoxon signed rank test, based on 5,000 replications.

3 Results

Genetic structure

The eight enzyme systems used showed 10 loci that could be interpreted and 20 alleles. The phenotypic expression of the enzymes was compatible with the typical monomeric loci pattern and segregating two alleles of the locus, following the Mendelian segregation model. No exclusive alleles were detected by the analyses of the allele frequencies of the 10 polymorphic loci in the five fragments and the vegetation corridor studied, because both alleles were present in all populations (Table 2). No major allele frequency difference was detected among fragments F1, F2 and F5 and the corridors axis F1-F2 and F4-F5, resulting in genetic equity (*EG*) for more than 50% of loci.

A positive correlation was observed between genetic equity and gene diversity for the fragments (*r* de Pearson = 0.957, R^2 = 0.915; d.f. = 4) and corridors (*r* de Pearson = 0.997, R^2 = 0.995; d.f. = 3). The relationship between the observed ($\hat{H}_o$) and expected ($\hat{H}_e$) mean heterozygosities resulted in a negative

fixation index ($\hat{f}$) in all the fragments analyzed, indicating a greater proportion of heterozygotes. This index was significant for fragments F3 and F5, indicating the H–W deviations. The mean estimated $\hat{H}_e$ values on the loci were significant for the fragments ($F_{ANOVA} = 20.79$; $P < 0.05$) and the corridors axis ($F_{ANOVA} = 8.65$; $P < 0.05$). All comparisons with fragment F3 and the comparisons F1 *x* F4 and F4 *x* F5 were significant after Bonferroni correction ($\alpha = 0.005$). Significant differences were observed in the corridor axis among the F1-F2 *x* F3-F4, F2-F3 *x* F4-F5 and F3-F4 *x* F4-F5. A positive correlation was observed between gene diversity and plant density in the fragments (*r* de Pearson = 0.742; $R^2 = 0.551$; d.f. = 4).

The mean estimates obtained for the coancestry coefficient (Cockerham 1969) were negative and significant, showing excess heterozygosity compared to that expected under H–W proportions (Table 3). The estimates indicated the absence of inbreeding for the set of the fragments ($\hat{F}$) and a tendency to present a greater number of heterozygotes within of the fragments ($\hat{f}$), suggesting that, on average, the populations in the fragments and corridors were not inbreeding. The population differentiation for the fragment-corridor system was low ($\hat{\theta}_p$ = 0,028). This meant that approximately 2.8% of the genetic variability was found among the fragments and that 97.2% of this variability occurred within the fragments. Generally, the species presented loci adhering to H–W proportions in some fragments. Significant deviations from the equilibrium model were detected only in the *Mdh-1* ($P = 0.012$) locus of fragment F2 and the *Adh* ($P = 0.021$) and *Mdh-2* ($P = 0.025$) locus on fragment F5. The significant deviations resulted from the excess heterozygosity verified in these loci.

The lowest genetic identity was observed among the individuals sampled in fragment F5 and those from axis F3, F2-F3 and F3-F4, with significant values (Figure 2). The cophenetic correlation coefficient, which assesses the existence of the clustering obtained, was of $r_C = 0.70$.

Minimum viable populations and bottlenecks

To plan *in situ* conservation, the $\hat{N}_{e(\,reference\,)}$ /($\hat{N}_e$ /*N*) ratios permitted estimation of the minimum viable population size (*PMV*), corresponding to the minimum number of trees that should be maintained or that would be necessary to ensure the maintenance of the genetic variability levels in the fragments. Therefore, the $\hat{N}_{e(\,reference\,)}$ of 150 and 1500, proposed by Nunney and Campbell (1993), for short and long-term conservation, respectively, was used as reference. For example, considering *PMV* calculated for F3 of 113 individuals for short term conservation and the estimates of 98 individuals in the fragment

[(11.8 ha)(8.3 ind.ha^{-1})], the difference was $\hat{D}$ = -15 individuals. Only fragment F5, because of the greater heterozygosity, did not present *PMV* deficit for conservation in the short or long term.

From the tests of fit to the infinite alleles model of mutation (Table 4), no population in the fragments was shown to be in equilibrium, indicating recent bottlenecks ($P < 0.05$, Wilcoxon sign-rank test). In all fragments, the populations showed a significant number of loci with excess heterozygosity, that is, the heterozygosity from the H–W proportions ($\hat{H}_e$) in the polymorphic loci was greater than the expected heterozygosity under equilibrium between mutation and drift ($\hat{H}_{eq}$).

4 Discussion

Genetic diversity

Our findings show that populations of *Protium spruceanum* in vegetation fragments and corridors maintain high levels of allozyme diversity. The similarity in the allele frequencies in fragments F1, F2 and F5, together with the similar genetic equity proportions, suggested low divergence among these fragments. The genetic equity is the least variation in the allele frequencies in the species, and is therefore an indication of greater genetic diversity (Frankel et al. 1995). However, given the longevity of most tree species, the study of the next generations will be required to provide a clear picture of the genetic fate of the studied populations.

The high proportion of polymorphic loci and the number of alleles per locus detected here were similar to reports for other tree species in investigation about impacts of habitat degradation on genetic resources, using allozymic markers (Bacles et al. 2004; Hall et al. 1996; Fuchs et al. 2003). The gene diversity ($\hat{H}_e$) detected for the species in the fragments and corridors was greater than the value estimated for tree species in general (0.17, Hamrick and Godt 1989), and can be explained by the absence of rare alleles, allele frequencies in equity, functionally unisexual flowers and high population density. The positive correlation between gene diversity and plant density was probably due to the reproductive system of the species. The outcrossing rates in tropical tree species can have an impact on the genetic structure (Murawski and Hamrick 1991; Nason and Hamrick 1997) and can result from ecological factors, population size and density (Franceschinelli and Bawa 2000; Murawski and Hamrick 1991). The plant's floral neighborhood (Feinsinger et al. 1986) in one area has a great effect on the plant reproductive success, because individuals in larger aggregations can attract more or better pollinators (Calvo and Horvitz 1990; Corbet 1998). In this context, the species in the fragments studied presented high demographic density. Thus the greater number of reproductive individuals

probably favored the increase in the levels of genetic diversity in function of the increases in the outcrossing rates, as discussed by van Treuren et al. (1993) and Franceschinelli and Bawa (2000).

The genetic differentiation detected in the study (2.8%) is in line with that reported for other tropical tree species, that is, a greater proportion of the genetic variability was found within the populations (Hall et al. 1996; Dayanandan et al. 1999; White et al. 1999). The typically outcrossing species presented high genetic variability within populations (Loveless and Hamrick 1984). Furthermore, the allele frequencies were generally equally distributed in the fragments and were thus an indication of gene flow acting as homogenizer of these frequencies, contributing to lower genetic differentiation observed among the fragments. The pattern of genetic identity found among the fragments and corridors may be associated to the gene flow among them. However, the genetic effects on tree species in anthropogenic forest fragments studied might be all less than 200 years old, a period, that may be insufficient for genetic changes to develop. Additional studies of direct methods (DNA-based) for estimating contemporary gene flow across the landscape are necessary to provide a clear picture of the contribution of seed and pollen to the overall contemporary gene immigration. Investigations on contemporary patterns of genetic structure within populations, e.g. at fine-scale spatial genetic structure, also is necessary. Nevertheless, considering the practically irreversible fragmentation of populations and the larger genetic diversity found in vegetation corridors, landscape management strategies should consider both the creation of new vegetation corridors and the protection of extant ones.

Effective population size and conservation applications

The estimate of $\hat{N}_e$ was associated with the conditions of heterozygosity and, therefore, the values observed reaffirmed the existence of low inbreeding in the fragments studied. $\hat{N}_e/N$ ratios are a very important parameter in germplasm preservation activities, seed collection and *in situ* genetic conservation, helping in genetic management and conservation projects. Data on the genetic representativeness of the population mother trees ($\hat{N}_e/N$) are important to maximize seed collecting activities (Vencovsky 1997). According to the $\hat{N}_e/N$ ratios obtained, a larger sampling of mother plants in the fragments that present lower $\hat{N}_e/N$ ratios (e.g. F4) is recommended for seed collection to ensure the maintenance of the genetic variability and minimum inbreeding in the seeds. Furthermore, for seed collection and germplasm conservation, the sampling should be random, not of seeds but of the mother plants. In vegetation enrichment, area recovery or creation of new vegetation corridors, seed

collection based on this principle will provide new genetic recombinations in the population and raise the evolutionary potential (Vencovsky 1997). The results of this will be important, because the species is indicated for use in the recovery of degraded areas. The species can be used especially in the recovery of riparian woods because of its abundant occurrence in the study region and its ecological adaptation to wet environments, along with fast growth and abundant seed production.

The determination of the *PMV* is, generally, performed without previous definition of when the conservation of the population under study will be carried out. Thus, the forest remnants studied gave important references for short and long-term conservation. The suitable values of *PMV* to be adopted for genetic conservation were determined by mainly two criteria: prevention of inbreeding depression and maintaining the evolutionary potential (Nunney and Campbell 1993). Thus maintenance of the suggested *PMV* will decrease the likelihood of genetic oscillations and inbreeding (Ellstrand and Elam 1993; Nunney and Campbell 1993). Our results showed that except for fragment F3, the species presented possibilities of maintaining its genetic equilibrium diversity in the short term, because the difference ($\hat{D}$) between the estimated population size ($\hat{N}$) and *PMV* was positive. Only fragment F5 presented possibilities of maintaining its genetic equilibrium diversity in the long term, that is, not present individual deficit for *PMV* for conservation in the long term. Corridor maintenance and conservation would then be plausible figures to ensure the demographic dynamics and higher neighborhood rates (gene flow by insect-pollinated and bird-dispersed) in the fragments, increasing the effective population sizes.

Recent bottlenecks

Recent decreases in $\hat{N}_e$ have been analyzed in the genetic study of populations (Bacles et al. 2004; Jump and Peñuelas 2006). Here, we also found a significant excess of H–W expected heterozygosity under both the infinite allele and stepwise mutation models, indicating the occurrence of recent population bottlenecks in all the fragments. Populations in mutation–drift equilibrium of the number of alleles present equal likelihood of a locus presenting an excess or a deficit of heterozygosity, considering an $\hat{N}_e$ that has remained constant in a recent past. In populations that have suffered recent drift, most of the loci will exhibit an excess of H–W expected heterozygosity (Luikart and Cornuet 1998), a situation that was presented in the fragments analyzed here. Thus, in a population recently reduced in population size, the genetic diversity observed will be greater than the genetic diversity equilibrium. The detection of recent bottlenecks also corroborates historical evidence that the forest fragments were

once part of a much larger population, and can be interpreted as a consequence of the habitat fragmentation resulting from human disturbance – from the Brazilian colonization period, two centuries a go.

The detection of populations that have undergone recent bottlenecks is important mainly because it allows inference of the risk of local extinction in consequence of the reduced population size (Lee et al. 2002). After detecting a bottleneck, the likelihood of the deleterious effects being avoided or minimized is greater because mitigating management procedures or immigrant introduction can be carried out (Luikart et al. 1998). Furthermore, these practices can be effective if associated with knowledge of ecological and demographic factors of the species. Thus, considering the recent genetic bottleneck detected, the lower genetic diversity and population size in fragment F3, and the practically irreversible situation of the habitat fragmentation, conservation of the vegetation corridors (e.g. F2-F3 and F3-F4) is a plausible option to maintain the number of migrants.

Management and conservation genetics

The understanding of the survival mechanisms of populations threatened by fragmentation of the ecosystems is the focus of the genetic conservation studies (Bacles et al. 2004; Cascante et al. 2002; Hall et al. 1996; Nason and Hamrick 1997; Templeton et al. 1990; White et al. 2002), and assessment of the consequences of reducing and isolating populations is fundamental for predicting the destiny of species in forest fragments for effective planning of management programs in small forest areas (Young and Clarke 2000). In population conservation and management programs, it is important to note the variations in genetic diversity caused by ecological and anthropological factors (Dick et al. 2003; Bacles et al. 2004). In this context, it is important to understand the landscape structure and the species biology, and conservation strategies based on species models can contribute to the conservation of entire ecosystems (Bacles et al. 2006, Jump and Peñuelas 2006). Here, we studied a mass-flowering insect-pollinated and bird-dispersed tropical tree. In the same place, other tree species with the similar life history characteristics, such as *Tapirira guianensis*, *Copaifera langsdorffii* and *Ocotea pulchella* are the most important and abundant species (Castro 2004), besides the congeneric species *Protium widgrenii* and *Protium heptaphyllum*. *Tapirira guianensis* is the most abundant species and present massive annual flowering with quick and well synchronized blooming peaks of both male and female individuals (Lenza and Oliveira 2005), similar reproductive traits to the one of *Protium spruceanum*. In conclusion, the high genetic diversity compared to the mean observed in other tropical tree species (Hamrick and Godt 1989) suggested a high potential for *in situ* genetic conservation and for seed collection destined to restore degraded

areas, including for the creation of new vegetation corridors. Thus, our study suggests that creation and the protection of vegetation corridors should be an effective conservation strategy for this tree species. That would be an important alternative to the demographic and genetic connection of isolated forest remnants, thus minimizing the negative effects of habitat fragmentation (Andreassen et al. 1998; Mech and Hallet 2001). In this sense, the vegetation corridors studied presented high gene diversity, genetic identity with the fragments and the absence of inbreeding. Therefore, to reduce the likelihood of deleterious effects from the bottleneck detected, vegetation corridors conservation would be a plausible option to maintain the number of migrants. These results give important data for species conservation. Furthermore, data on the reproductive biology, regeneration and genetic distribution pattern of the different forest species can contribute to the conservation strategies of the small forest remnants.

Acknowledgements
The authors acknowledge Anderson M. de Souza for field assistance, Dr. Eduardo L. Borba (UFMG-Brazil) and Dr. Christian Lexer (RBG-Kew) and anonymous referees for helpful and constructive comments on the manuscript. Gislene C. de Castro for providing information on plant density and Vivette Appolinário helped in the collection and analysis of reproductive biology data. The Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Conselho Nacional Pesquisa (CNPq) are also acknowledged for providing a MSc fellowship for F.A. Vieira, a research fellowship for Dr. Dulcinéia de Carvalho and research grant. We thank the FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais) for financial support (CRA 1770/05).

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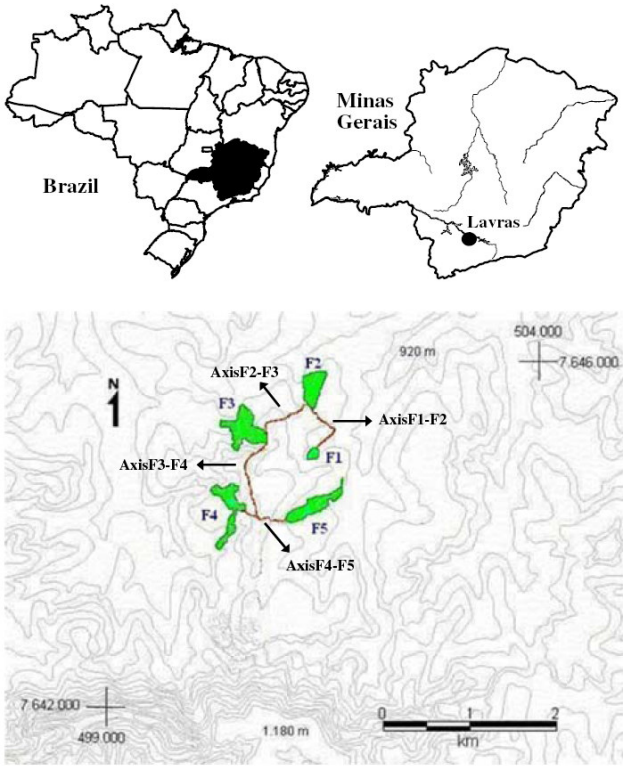

Fig. 1 Location of the study system with primary forest remnants and secondary vegetation corridors in Minas Gerais state, Brazil. F1–F5 (fragments) and Axis F1-F2 to F4-F5 (vegetation corridors). The coordinates are according to the Universal Transverse Mercator (UTM) system

Table 1 Fragments and corridor codes, altitude and area of the fragments, sample size (*N*) and density of the species (*DA*) sampled in this study

Code	Fragments					Corridors			
	F1	F2	F3	F4	F5	F1-F2	F2-F3	F3-F4	F4-F5
Altitude (m)	973.0	969.8	984.3	985.0	986.0				
Area (ha)	1.0	7.2	11.8	7.4	7.8	380[a]	450	510	590
N	30	30	30	30	30	20	20	20	20
DA (ind ha^{-1})	850.0	50.0	8.3	175.0	175.0				

In the vegetation corridor the absolute density of the species is 135.19 ind ha^{-1} (Castro 2004)

[a] Extension (m) of the vegetation corridors

Table 2 Levels and distribution of genetic diversity of a mass-flowering insect-pollinated and bird-dispersed tree (*P. spruceanum*) in fragment-corridor system

	Fragments					Corridor axis			
	F1	F2	F3	F4	F5	F1-F2	F2-F3	F3-F4	F4-F5
EG (%)	90	80	20	30	100	60	30	20	70
$\hat{A}$	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
$\hat{P}$	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
$\hat{H}_e$(SD)	0.480 (0.016)	0.469 (0.015)	0.381 (0.065)	0.437 (0.027)	0.507 (0.002)	0.454 (0.050)	0.383 (0.071)	0.336 (0.094)	0.470 (0.041)
$\hat{f}$	−0.170	−0.182	−0.250*	−0.093	−0.248*	0.078	−0.023	−0.123	−0.029
Loci	Allele /*N*								
Adh	1	0.617	0.667	0.783	0.700	0.482	0.725	0.825	0.825	0.600
	2	0.383	0.333	0.217	0.300	0.518	0.275	0.175	0.175	0.400
	N	30	30	30	30	28	20	20	20	20
Gdh	1	0.603	0.650	0.767	0.717	0.533	0.684	0.775	0.816	0.579
	2	0.397	0.350	0.233	0.283	0.467	0.316	0.225	0.184	0.421
	N	29	30	30	30	30	19	20	19	19
Gldh	1	0.603	0.643	0.810	0.683	0.500	0.650	0.775	0.825	0.575
	2	0.397	0.357	0.190	0.317	0.500	0.350	0.225	0.175	0.425
	N	29	28	29	30	29	20	20	20	20
Gtdh	1	0.617	0.650	0.776	0.650	0.517	0.725	0.800	0.850	0.600
	2	0.383	0.350	0.224	0.350	0.483	0.275	0.200	0.150	0.400
	N	30	30	29	30	30	20	20	20	20
Mdh-1	1	0.650	0.567	0.517	0.600	0.533	0.500	0.625	0.550	0.750
	2	0.350	0.433	0.483	0.400	0.467	0.500	0.375	0.450	0.250
	N	30	30	29	30	30	20	20	20	20

Table 2 continued

Loci	Allele /*N*									
Mdh-2	1	0.586	0.650	0.783	0.717	0.467	0.778	0.750	0.825	0.625
	2	0.414	0.350	0.217	0.283	0.533	0.222	0.250	0.175	0.375
	N	29	30	30	30	30	18	20	20	20
Per-1	1	0.683	0.600	0.583	0.683	0.550	0.625	0.650	0.816	0.675
	2	0.317	0.400	0.417	0.317	0.450	0.375	0.350	0.184	0.325
	N	30	30	30	30	30	20	20	19	20
Per-2	1	0.617	0.650	0.783	0.650	0.533	0.525	0.600	0.550	0.725
	2	0.383	0.350	0.217	0.350	0.467	0.475	0.400	0.450	0.275
	N	30	30	30	30	30	20	20	20	20
Sdh	1	0.600	0.667	0.767	0.733	0.466	0.650	0.800	0.800	0.600
	2	0.400	0.333	0.233	0.267	0.534	0.350	0.200	0.200	0.400
	N	30	30	30	30	29	20	20	20	20
Skdh	1	0.583	0.621	0.750	0.655	0.536	0.625	0.789	0.861	0.600
	2	0.417	0.379	0.250	0.345	0.464	0.375	0.211	0.139	0.400
	N	30	29	30	29	28	20	19	18	20

* $P < 0.05$; EG, genetic equity; $\hat{A}$, mean number of alleles per locus; $\hat{P}$, percentage of polymorphic loci; $\hat{H}_e$, Nei's gene diversity; SD, standard deviations; $\hat{f}$, mean fixation index

Table 3 Estimates of Wright's *F*-statistics described for each polymorphic locus in *Protium spruceanum*

Loci	$\hat{f}$	$\hat{F}$	$\hat{\theta}_p$
Adh	−0.158	−0.109	0.042
Gdh	−0.121	−0.094	0.024
Gldh	−0.130	−0.087	0.038
Gtdh	−0.110	−0.072	0.034
Mdh-1	−0.174	−0.166	0.006
Mdh-2	−0.140	−0.087	0.046
Per-1	−0.035	−0.029	0.006
Per-2	−0.138	−0.117	0.018
Sdh	−0.177	−0.129	0.041
Skdh	−0.145	−0.112	0.029
Mean	−0.133*	−0.101*	0.028*
	[−0.153 to −0.108]	[−0.122 to −0.079]	[0.019 to 0.037]

* $P < 0.05$; $\hat{f}$, mean fixation index of individuals relative to their population; $\hat{F}$, mean overall inbreeding coefficient of an individual; $\hat{\theta}_p$, populations coancestry coefficient; [ ], confidence intervals

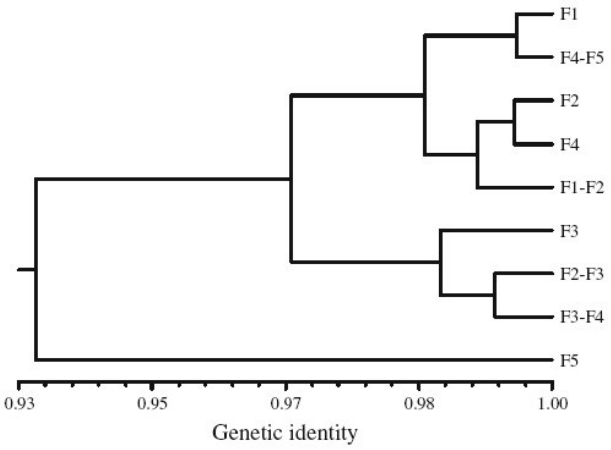

Fig. 2 Dendrogram constructed according to values of Nei's genetic identity (UPGMA) found for *P. spruceanum* in five fragments and vegetation corridors axis

Table 4 Number of loci showing deficiency/excess of heterozygosity under IAM for bottleneck detection in *Protium spruceanum*

	Fragments				
	F1	F2	F3	F4	F5
Expected number of loci showing excess of heterozygosity	3.8	4.2	4.4	4.3	3.4
Deficiency/excess of heterozygosity	0/9*	0/10*	0/10*	0/10*	0/8*
$\hat{N}_e/N$	1.20	1.22	1.33	1.10	1.33
$\hat{N}$	850	360	98	1,295	1,365
PMV (150)	125	123	113	136	113
$\hat{D}(150)$	725	237	−15	1,159	1,252
PMV (1,500)	1,245	1,227	1,125	1,361	1,128
$\hat{D}(1,500)$	−395	−867	−1027	−66	237

* Significant as determined by Wilcoxon signed rank tests ($\alpha = 0.05$); $\hat{N}_e$, effective population size; N, sample size; $\hat{N}$, population size estimated; PMV, minimum viable populations; (150), conservation in the short term; (1,500), conservation in the long term; $\hat{D}$, difference

ARTIGO 2

Genetic differentiation of the neotropical tree species *Protium spruceanum* (Benth.) Engler. (Burseraceae) between fragments and vegetation corridors in Brazilian Atlantic forest

(Preparado de acordo com as normas da *Acta Botanica Brasilica*)

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RESUMO – (Diferenciação genética da espécie arbórea neotropical *Protium spruceanum* (Benth.) Engler. (Burseraceae) entre fragmentos e corredores de vegetação em floresta Atlântica Brasileira). Foram estudados os padrões de diferenciação genética em uma paisagem conectada com uma interessante história de conversão humana do habitat, que iniciou há dois séculos durante o período colonial do Brasil. Nos fragmentos de floresta estacional Atlântica primária e corredores de floresta secundária, *Protium spruceanum* é uma arbórea nativa abundante, com floração massiva, polinizada por insetos e dispersa por pássaros. A distribuição da diversidade genética foi analisada por meio de locos aloenzimáticos em 230 indivíduos em cinco fragmentos (1 a 11,8 ha) e quatro corredores (460 a 1000 m). Foi observada ausência de endogamia nos fragmentos e corredores, mas a proporção de heterozigotos ($\hat{f}$) foi significativamente maior nos fragmentos de floresta primária do que nos corredores de vegetação secundária, conforme teste *G* de Goudet ($P = 0.036$). A diferenciação genética foi baixa e nenhum padrão de isolamento pela distância foi observado. Observou-se, em geral, menor diferenciação genética entre fragmentos e corredores vizinhos, indicando possível fluxo gênico por sementes e pólen. Assim, conclui-se que os corredores de floresta secundária podem ser uma alternativa na conexão genética de fragmentos isolados. Isto é assim consistente com a baixa diferenciação observada entre eles e na ausência de uma redução significante da diversidade genética nos corredores de floresta secundária.

Palavras-chave: aloenzimas, corredores de vegetação, diversidade genética, estatística-*F*, fragmentação de habitat

ABSTRACT – (Genetic differentiation of the neotropical tree species *Protium spruceanum* (Benth.) Engler. (Burseraceae) between fragments and vegetation corridors in Brazilian Atlantic forest). We studied patterns of genetic differentiation in a connected landscape with an interesting history of human habitat conversion that began two centuries ago, during Brazilian colonization period. In the fragments of undisturbed Brazilian Atlantic seasonal forest and corridors of secondary forest, *Protium spruceanum* is an abundant native, mass-flowering/insect-pollinated and bird-dispersed tree. Genetic diversity was analised from 230 individuals in five fragments (1 to 11.8 ha) and four corridors

(460 to 1000 m length) using allozyme loci. We did not find evidence of inbreeding within fragments or corridors, but the proportion of heterozygotes ($\hat{f}$) were significantly higher in undisturbed primary fragments than in the secondary vegetation corridors, based on Goudet's *G*-test ($P = 0.036$). Genetic differentiation was low and no pattern of isolation by distance was found. All fragments generally have lower historical genetic differentiation with corridors which they are connected, indicating possible gene flow via seeds and pollen. We conclude that corridors of second-growth forests would be an important alternative in the genetic connection of isolated forest fragments. This is thus consistent with the low differentiation observed among them and the absence of a significant reduction in gene diversity in second-growth forests.

Key words: allozymes, *F*-statistics, genetic diversity, habitat fragmentation, vegetation corridors

1 Introduction

Many studies have reported the spatial patterns of genetic variation in a range of species with different life history characteristics and associated with landscape features, especially those related to recent human occupation (Manel *et al.* 2003; Lowe *et al.* 2005), to provide information on how landscape and environmental features influence population genetic structure (Storfer *et al*. 2007). Possible theoretical impacts generated by different types of human activity would suggest that forest loss and spatial isolation of natural populations can decrease levels of gene flow and reduce effective population size of a species in a region (Fahring 2003). If the remaining population is isolated for many generations, forest fragmentation may lead to continuous allele loss (Aldrich & Hamrick 1998; Couvet 2002) and consequently, there will be an increase in the genetic divergence between these more isolated populations in the region (Pither *et al*. 2003). In contrast, some studies have shown that habitat fragmentation facilitated both pollen movement (White *et al.* 2002; Dick *et al.* 2003) and long-distance dispersal (Bacles *et al.* 2006).

Studies at landscape-level scales provide insight into micro-evolutionary patterns by elucidating the movement of genes at a range of spatial scales (Manel *et al.* 2003; Storfer *et al*. 2007). Several studies have evaluated the

landscape context between undisturbed primary forest and secondary forest (Aldrich & Hamrick 1998; Hamilton 1999; Sezen *et al.* 2005; Ally & Ritland 2007), but few works have estimated the role of corridors and landscape connectivity for plants (Debinski & Holt 2000), instead tending to focus primarily on genetic processes (Kirchner *et al*. 2003). For second-growth forests, population genetic studies indicate bottlenecks, through reproductive dominance, reduced genetic diversity of a founder population and increased levels of inbreeding (Aldrich & Hamrick 1998; Sezen *et al.* 2005). Thus, tree populations in second-growth forests will require continuous gene flow over successive generations to restore genetic diversity to levels currently observed in older more established forests (Sezen *et al.* 2005). Alternatively, corridors have been proposed as one way to mediate the effects of habitat fragmentation on populations (Beier & Noss 1998; Nasi *et al*. 2008). Some studies have provided convincing evidence that, in some cases, corridors can enhance migration rates among fragments (Aars & Ims 1999; Mech & Hallet 2001).

Here we examine the patterns of genetic differentiation in a landscape with an interesting history of human habitat conversion. The Brazilian Atlantic forest, in southern Minas Gerais state, has been seriously exploited since European occupation two centuries ago. This exploitation resulted in the fragmentation and isolation of these populations at a particularly rapid pace. The detection of recent genetic bottlenecks also corroborates historical evidence that the forest fragments were once part of a much larger population and can be interpreted as a consequence of the habitat fragmentation resulting from the Brazilian colonization period (Vieira & Carvalho 2008). At the same time, ditches to divide rural properties (≈ 6 m-wide) were constructed by slaves, resulting in the vegetation corridors, that is, second-growth colonization by native tree species that connect small fragments of undisturbed primary forest.

Where fragments and vegetation corridor areas coexist, the historic fragmentation of these plant formations provides the opportunity to assess the effects of chronic habitat fragmentation and the influence of vegetation corridors. For that, we used analysis of allozymes, which have been successfully used to address the questions about genetic effects of habitat fragmentation on tree species (Hall *et al.* 1996; Fuchs *et al.* 2003; Franceschinelli *et al.* 2007; Martins *et al*. 2008). *Protium spruceanum* was selected for this research as highly representative of a broad range of taxa with common suite of ecological characteristics for tropical trees in the study region: high populational density, mass-flowering, insect-pollinated and bird-dispersed tree species. We

hypothesized that patterns of genetic variation in fragments should reflect the expectation for a species that is typically outcrossing, namely high levels of genetic variation within and relatively low levels of differentiation between fragment populations. If seed dispersal and pollen movement is widespread relative to the distance between fragments the result should be a lack of detected spatial genetic structure by isolation by distance model. Finally, theoretical predictions suggest reduced genetic diversity and increased levels of inbreeding in corridors of second-growth forests.

2 Materials and methods

Study species – *Protium spruceanum* (Benth.) Engler (Burseraceae) is a large canopy tree (up to 25 m tall), found in the Amazon and the Atlantic rain forests and on the cerrado inside riverbank woodland (Oliveira Filho & Ratter 1995). The recruitment of seedlings may occur under large trees, since *P. spruceanum* is shade-tolerant. The small, pale yellowish flowers (0.3-0.4 cm wide) are functionally unisexual and organized in dense inflorescences (with ca. 45 flowers) and the individuals are dioecious. The effective pollinators are *Apis mellifera* and *Trigona* sp. (Hymenoptera, Apidae) (F. A. Vieira *et al*., unpublished data, 2009). The medium-sized, bird-dispersed seeds (< 500 mg fresh weight) are produced in reddish berries in the canopy of adult trees and are dispersed from October to March, with a peak in November (F. A. Vieira *et al*., unpublished data, 2009). In the fragment-corridor system studied, *P. spruceanum*, along with companion tree species such as *Tapirira guianensis* Aublet (Anacardiaceae), *Copaifera langsdorffii* Desf. (Fabaceae), *Ocotea pulchella* Mart. (Lauraceae) and the congeners *P. widgrenii* Engler and *P. heptaphyllum* (Aublet) Marchand (Burseraceae), represent the most abundant species of a mass-flowering/insect-pollinated and bird-dispersed tree species.

Study site and sampling design – The fragment-corridor system studied here is located in the region of Lavras, South of Minas Gerais state, in Brazil (Fig. 1). In the current landscape a limited number of fragments of primary forest, a matrix of planted pastures and vegetation corridors of secondary forest can be observed. The populations studied have rapidly declined because of habitat fragmentation caused by anthropogenic disturbance over the last 200 y (Vieira & Carvalho, 2008). Thus, the estimated age of the trees is of 2–4 generations before present, assuming a generation time of 50–100 y. Five interlinking fragments and a

vegetation corridor were analyzed (Tab. 1). *P. spruceanum* presence in fragments F2, F3 and F4 coincides with the presence of water courses, in F1 and F5 the species occurs in a large area of the fragment, which may be attributed to semi-permanently flooded soil. All samples came from trees with diameter at breast height (dbh) > 20 cm and ≈ 16 m in height from the interior of each fragment within an area of about 1 to 11.8 ha. The sampling in each corridor was along the length of each corridor axis of about 460 to 1000 m.

Enzyme extraction and electrophoresis – Enzymes were extracted from 200 mg of frozen leaf tissue in 1 mL of phosphate extraction buffer: 0.2 mM sucrose, 2.5% polyvinylpyrrolidone (PVP), 1 mM ethylenediaminetetraacetic acid (EDTA), 5.7 mM ascorbic acid, 5.8 mM sodium diethyl carbamate (DIECA), 2.6 mM sodium bisulphite, 2.5 mM Borax and 0.2% β-mercaptoethanol. Discontinuous vertical electrophoresis in a polyacrylamide gel was performed using 10% page gels and carried out at 4 °C over 3 h (constant current of 80 mA, and voltage of 300 V). Eight enzymatic systems were used: alcohol dehydrogenase (E.C.1.1.1.1, locus *Adh*), glucose dehydrogenase (E.C.1.1.1.47, locus *Gdh*), β-galactose dehydrogenase (E.C.1.1.1.48, locus *Gldh*), glutamate dehydrogenase (E.C.1.4.1.3, locus *Gtdh*), malate dehydrogenase (E.C.1.1.1.37, loci *Mdh-1* and *Mdh-2*), peroxidase (E.C.1.11.1.7, loci *Per-1* and *Per-2*), sorbitol dehydrogenase (E.C.1.1.1.14, locus *Sdh*) and shikimate dehydrogenase (E.C.1.1.1.25, locus *Skdh*). Staining protocols and the genetic basis of allozymes banding patterns were inferred from segregation patterns with reference to typical subunit structure and conceptual methods (Wendel & Weeden 1989). Putative loci and alleles were designated sequentially. The most anodally migrating allozyme or alleles was designated as *1* and the next as *2*.

Data analyses – The genetic variation within each population was estimated by the proportion of polymorphic loci (P_L; 0.95 criterion), mean number of alleles per locus ($\hat{A}$), observed heterozygosity ($\hat{H}_o$) and Nei's gene diversity ($\hat{H}_e$). An estimate of inbreeding levels for each population was obtained using Wright's fixation index ($\hat{f} = 1 - \hat{H}_o/\hat{H}_e$). All these parameters were calculated using BIOSYS 2 (Swofford & Selander 1989) and GDA programs (Lewis & Zaykin 2001). Wright's *F*-statistics ($\hat{f}$ and $\hat{F}$, Wright 1943) were used to measure hierarchical population structure and were calculated by the methods of Weir and Cockerham (1984). Confidence intervals at 95% probability were

established for each population using the bootstrap procedure with 10000 repetitions. These parameters were estimated using the program GDA. Comparisons among fragments and corridors according to these parameters ($\hat{H}_o$, $\hat{H}_e$ and $\hat{f}$), were made using the program FSTAT 2.9.3.2 (Goudet 2002), through the *G*-based exact test by randomization of multilocus genotypes for allozymes proposed by Goudet *et al*. (1996).

We calculated pairwise differentiation between fragments and fragments and corridors using $\hat{F}_{ST}$ statistics (Weir & Cockerham 1984). Pairwise tests of differentiation were performed using the *G*-test, based on 3600 permutations of genotypes among samples (Goudet *et al.* 1996). Significance tests of multilocus pairwise $\hat{F}_{ST}$ were carried out using the program FSTAT with standard Bonferroni corrections. To test for isolation by distance, pairwise ($\hat{F}_{ST}$ /1– $\hat{F}_{ST}$) matrices were related to geographical distances between fragments. Mantel tests were used to test for significance (1000 permutations) with the software NTSYS 1.5 program (Rohlf 1989).

3 Results

Genetic diversity – Ten polymorphic loci and twenty alleles were observed and analyzed. The percentage of polymorphic loci (P_L = 100%) and average number of alleles per locus ($\hat{A}$ = 2.0) were similar for all populations. The negative Wright's *F*-statistics indicated the absence of inbreeding for the fragments and corridors (Tab. 2). The relationship between the observed $\hat{H}_o$ and expected $\hat{H}_e$ mean heterozygosities resulted in a significant heterozygosity excess $\hat{f}$ for fragments (Tab. 2). Based on Goudet's *G*-test, the mean value of observed heterozygosities (P = 0.044) and fixation index (P = 0.036) were significantly higher in fragments than corridors. The mean value of gene diversity was not significantly different between fragments and corridors (P = 0.242).

Genetic differentiation between fragments and corridors – Specific genetic differentiation was low, the F_{ST} value of 0.028 suggesting that only 2.8% of the genetic variability is distributed between fragments, and that 97.2% of such variability occurs within fragments. The genetic differentiation in each forest fragment pair (Tab. 3) and for each fragment and corridor pair (Tab. 4) was generally low. Between primary forest fragments, the larger genetic

differentiation was between F3 and F5, corroborated by significant tests of population differentiation pairwise ($\hat{F}_{ST}$ = 0.111, $P < 0.001$) after Bonferroni correction (Tab. 3). Fragment F3 revealed low genetic differentiation with fragment F2 (these two were connected by the F2-F3 corridor). Indeed, fragment F3 has lower genetic differentiation exactly with the F2-F3 axis (Tab. 4). All fragments generally have lower genetic differentiation with corridors which they are connected. The highest observed genetic differentiation occurred between fragment F5 and F2-F3 ($\hat{F}_{ST}$ = 10.5%, $P < 0.001$) and F3-F4 axis ($\hat{F}_{ST}$ = 14.8%, $P < 0.001$), at 5% level. Mantel tests provided no evidence for distance dependence of genetic structure ($r_m = -0.051$, $P = 0.539$).

4 Discussion

Genetic diversity in *Protium spruceanum* – The number of alleles per locus ($\hat{A}$) was close compared with values of other tropical plants. Hamrick & Godt (1989), in a review of 653 genetic diversity studies using allozymes, found the following values of $\hat{A}$: 2.19 for woody long-lived species, 2.29 for widely distributed species, 1.81 for tropical species and 1.99 for allogamous animal-pollinated species. The gene diversity $\hat{H}_e$ detected within primary forest fragments and secondary vegetation corridors was greater than the value estimated for tree species in general ($\hat{H}_e$ = 0.17, Hamrick & Godt 1989) and is higher than that estimated for some tropical tree species, in recent studies using allozymes markers ($\hat{H}_e$ = 0.25 for *Acacia macracantha* and $\hat{H}_e$ = 0.24 for *A. aroma*, Casiva *et al.* 2004; $\hat{H}_e$ = 0.13 for *Pithecellobium elegans*, Hall *et al.* 1996), but is close to the other tropical tree species ($\hat{H}_e$ = 0.40 for *Pachira quinata*, Fuchs *et al.* 2003; $\hat{H}_e$ = 0.49 for *Shorea leprosula*, Ng *et al.* 2004). This high gene diversity found is likely to be associated with the reproductive system and demography of populations (Murawski & Hamrick 1991; Nason & Hamrick 1997). For *P. spruceanum*, the functionally unisexual flowers in different individuals flower synchronously, favouring outcrossing. Indeed, the population size effect on population genetic diversity might be pronounced mainly in outcrossing species (Honnay & Jacquemyn 2007) and outcrossing plants in general exhibit higher levels of gene diversity than selfing plants (Hamrick & Godt 1996). Density has also been observed to influence rates of outright outcrossing in animal-pollinated plant species showing a positive association (Loveless & Hamrick 1984; van Treuren *et al.* 1993). Moreover, compared with

endemic and rare taxa, widespread and abundant species often contain significantly more genetic variability (Cole 2003).

Although the mean value of gene diversity was not significantly different between fragments and corridors, through the *G*-based exact test, heterozygosities were significantly higher in fragments than secondary vegetation corridors. Thus, the estimates indicate that a greater proportion of heterozygotes are found in the undisturbed primary fragments than in the secondary vegetation corridors. These results are in line with theoretic predictions (Aldrich & Hamrick 1998; Sezen *et al.* 2005). However, given the longevity of most tree species, the study of the next generations will be required to provide a clear picture of the genetic fate of the studied populations.

Genetic differentiation – For an insect-pollinated and bird-dispersed tree, low genetic differentiation among primary forest fragments was expected and observed ($\hat{F}_{ST}$ = 0.028). This is in accordance with other tropical tree species, that is, higher genetic variability is detected within populations (Hall *et al.* 1996; Dayanandan *et al.* 1999). The low genetic differentiation estimated is probably the result, amongst other factors, of the population size and consequential massive annual flowering, featuring quick and well-synchronized blooming peaks (F. A. Vieira *et al*., unpublished data, 2009). Mass flowering represents the most extreme example of flowering synchrony at both the individual and population level (Frankie *et al.* 1974; Gentry 1978). Flowering synchrony can influence the levels of gene flow and differentiation among populations (Soliva & Widmer 1999) depending on the effects of other organisms such as pollinators and seed dispersers (Domínguez *et al*. 2005). High levels of synchrony at both the individual and population level may increase the number of pollinators attracted to flowers and the rate of outcrossing (Augspurger 1981), resulting in extensive pollen dispersal and gene flow (Byrne *et al*. 2007).

Although the amount of genetic differentiation found for *P. spruceanum* is consistent with that expected for outcrossing species (Hamrick & Godt 1996), it should be noted that genetic differentiation occurred among populations located at an average distance of only 1.2 km (0.81 to 2 km), and even those populations located as close as 1.38 km to each other (F3 and F5) showed significant differentiation. This result may be unexpected for a species that is an outcrosser, is pollinated by bees that are able to fly long distances and whose fruits are bird dispersed. Nevertheless, the genetic diversity levels may depend

on factors (e.g. population size, environmental heterogeneity, even random patterns) including the landscape structure (Manel *et al.* 2003). Thus, we combine genetic differentiation and distance between fragments, but no pattern of isolation by distance was found. Although all fragments generally have lower historical genetic differentiation with corridors which they are connected, indicating possible gene flow via seeds and pollen.

Conclusions and prospects – Changes in gene flow can be estimated by comparing 'historical' estimates based on genetic differentiation ($\hat{F}_{ST}$). Hence, indirect estimates of gene flow reflect historical rates of flow that have occurred over many generations (Loveless 1992). Typically outcrossing species display high within-population genetic diversity and low interpopulation genetic differentiation as a result of high interpopulation gene flow (Loveless & Hamrick 1984). Indeed, indirect estimates of gene flow revealed extensive gene exchange over the spatial scale of the study (F_{ST} = 2.8%), with high levels of genetic variation remaining across all fragments ($\hat{H}_e$ = 0.463). Nevertheless, considering the practically irreversible fragmentation of populations and the larger genetic diversity found in small undisturbed primary forests, landscape management strategies should consider the protection of extant ones. Small-sized forest fragments have been reported as habitats or stepping-stones for birds, and thus contribute to the connection of forest fragments (Estrada *et al.* 2000; Fischer & Lindenmayer 2002). In addition, new approaches to studying other species with different life history characteristics would be needed to investigate the functional aspect of connectivity as it relates to the biological response of the species to landscape structure. Investigations on contemporary patterns (DNA-based) of genetic structure within populations, e.g. at fine-scale spatial genetic structure, also is necessary. These current ideas and researches is now in progress. This paper gives us important baseline data that will be useful in future comparisons of other common insect-pollinated and bird-dispersed Atlantic forest tree species as well as comparisons of other fragmented forests that are not connected by corridors of natural vegetation.

Acknowledgements

The authors acknowledge Anderson M. de Souza for assistance on the collection of samples, Dr. Cecile F.E. Bacles (University of Queensland-

Australia), Dr. Henk W.M. Hilhorst (Wageningen University-The Netherlands) and Dr. Dion Devey (Royal Botanic Kew Gardens) for their helpful comments on the manuscript. The Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Conselho Nacional de Pesquisa (CNPq) are also ackowledged for providing a Dr. fellowship for F. A. Vieira, a research fellowship for Dr. Dulcinéia de Carvalho and research grant. This research was support by FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais).

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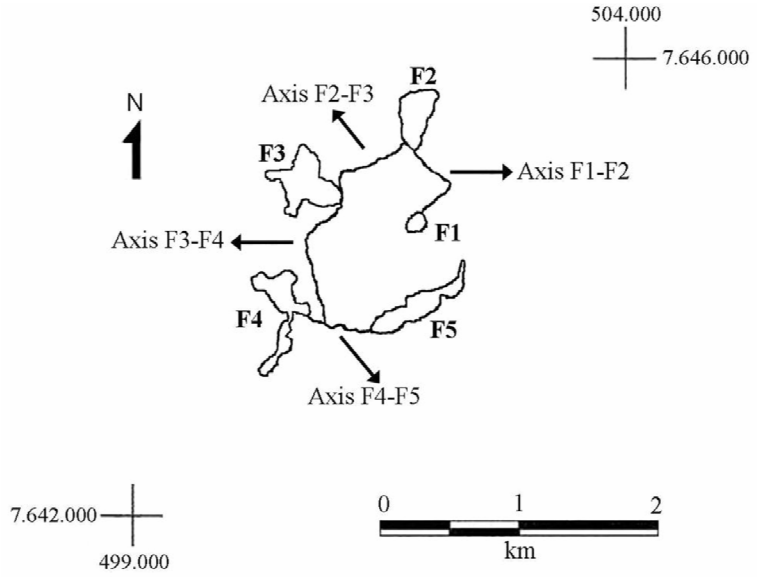

Figure 1. Location of the study system with forest fragments and secondary vegetation corridors in Minas Gerais state, Brazil. F1 to F5 (fragments) and Axis F1-F2 to F4-F5 (vegetation corridors). The coordinates are according to the Universal Transverse Mercator (UTM) system.

Table 1. Fragments and corridor codes, area of the fragments and length of the vegetation corridors, sample size (*N*), density of *Protium spruceanum* (Benth.) Engler. and genetic variation in populations sampled in this study. $\hat{H}_o$, mean observed heterozygosity (standard error); $\hat{H}_e$, mean gene diversity (standard error); $\hat{f}$, mean fixation index. In the vegetation corridor the absolute density of the species is 135 trees ha^{-1} (G. C. de Castro, unpublished data). * $P < 0.05$.

	Fragments					Corridors			
Code	F1	F2	F3	F4	F5	F1-F2	F2-F3	F3-F4	F4-F5
Area (ha)	1	7.2	11.8	7.4	7.8				
Length (m)						650	460	1000	540
N	30	30	30	30	30	20	20	20	20
Density ha^{-1}	850	50	8.3	175	175				
$\hat{H}_o$	0.559	0.552	0.475	0.477	0.630	0.419	0.392	0.376	0.483
	(0.070)	(0.069)	(0.071)	(0.038)	(0.072)	(0.106)	(0.100)	(0.103)	(0.044)
$\hat{H}_e$	0.480	0.469	0.381	0.437	0.507	0.454	0.383	0.336	0.470
	(0.016)	(0.015)	(0.065)	(0.027)	(0.002)	(0.050)	(0.071)	(0.094)	(0.041)
$\hat{f}$	–0.170	–0.182	–0.250*	–0.093	–0.248*	0.078	–0.023	–0.123	–0.029

Table 2. Allelic frequencies (allele *1*) for 10 allozyme loci and genetic diversity parameters for *Protium spruceanum* (Benth.) Engler. in the fragment-corridor system. $\hat{H}_o$, mean observed heterozygosity (standard error); $\hat{H}_e$, mean gene diversity (standard error); $\hat{f}$, mean fixation index; $\hat{F}$, mean overall inbreeding coefficient. * $P < 0.05$.

	Fragment	Corridor
Locus/Allelic frequencies		
Adh	0.650	0.744
Gdh	0.654	0.714
Gldh	0.648	0.706
Gtdh	0.642	0.744
Mdh-1	0.573	0.606
Mdh-2	0.641	0.744
Per-1	0.620	0.690
Per-2	0.647	0.600
Sdh	0.647	0.712
Skdh	0.629	0.714
Genetic diversity		
$\hat{H}_o$	0.538 (0.008)	0.418 (0.021)
$\hat{H}_e$	0.463 (0.004)	0.420 (0.012)
$\hat{f}$	–0.188*	–0.018
$\hat{F}$	–0.156*	–0.010

Table 3. Geographical distances (km, above diagonal) and genetic differentiation ($\hat{F}_{ST}$ values, below diagonal) among five fragments. * *P*-value < 0.001; NS, non-significant at 5% level.

Fragments	F1	F2	F3	F4	F5
F1	–	0.94	0.88	1.19	0.81
F2	0.007 ^{ns}	–	1.00	2.00	1.75
F3	0.048 ^{ns}	0.019 ^{ns}	–	1.25	1.38
F4	0.002 ^{ns}	0.008 ^{ns}	0.007 ^{ns}	–	0.81
F5	0.010 ^{ns}	0.024 ^{ns}	0.111*	0.053 ^{ns}	–

Table 4. Genetic differentiation ($\hat{F}_{ST}$) among forest fragments and secondary vegetation corridors. Estimates obtained and tests performed using FSTAT 2.9.3.2 (Goudet 2002). NS, non-significant at 5% level.

Fragments/corridors	$\hat{F}_{ST}$	P
F1 and F1-F2	0.003	NS
F1 and F2-F3	0.036	NS
F1 and F3-F4	0.071	< 0.01
F1 and F4-F5	0.013	NS
F2 and F1-F2	0.009	NS
F2 and F2-F3	0.015	NS
F2 and F3-F4	0.049	NS
F2 and F4-F5	0.004	NS
F3 and F1-F2	0.014	NS
F3 and F2-F3	0.003	NS
F3 and F3-F4	0.020	NS
F3 and F4-F5	0.051	NS
F4 and F1-F2	0.007	NS
F4 and F2-F3	0.001	NS
F4 and F3-F4	0.026	NS
F4 and F4-F5	0.002	NS
F5 and F1-F2	0.040	NS
F5 and F2-F3	0.105	< 0.001
F5 and F3-F4	0.148	< 0.001
F5 and F4-F5	0.018	NS
Overall	0.028	

ARTIGO 3

Reproductive biology of *Protium spruceanum* (Benth.) Engler. (Burseraceae), a dioecious-dominant tree in vegetation corridors in Southeastern Brazil

(Preparado para publicação na *Revista Brasileira de Botânica*)

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ABSTRACT – (Reproductive biology of *Protium spruceanum* (Benth.) Engler. (Burseraceae), a dioecious-dominant tree in vegetation corridors in Southeastern Brazil). *Protium spruceanum* is a large canopy tree (up to 20 m tall) occurring mainly in the Amazon, Atlantic rain forests and on the cerrado inside riverbank woodland. We investigated the reproductive biology of *P. spruceanum* in vegetation corridors of secondary Atlantic forest in Lavras, southern Minas Gerais, Brazil. The reproductive phenology was investigated fortnightly over a one year period. Floral biology studies involved pollen viability analysis, nectar production, stigmatic receptivity, pollen tube growth, visiting insect species and visit rates. The small, pale yellowish flowers (0.3–0.4 cm wide) are functionally unisexual and organized in dense inflorescences (with ca. 45 flowers). Reproductive activity takes place during the wet season. *P. spruceanum* presented abundant annual flowering of both male and female individuals with quick, synchronized blooming peaks, between September and November. Staminate flowers supplied a large quantity of viable pollen (90.6%) and relatively abundant nectar (4.5 µL). Pistillate flowers produced only nectar to flower visitors (4.0 µL). The effective pollinators were *Apis mellifera* and *Trigona* sp. (Hymenoptera, Apidae). Pollen tubes of cross-pollinated flowers were observed entering in the ovaries 48 h after pollination. *P. spruceanum* is characterized by a high rate of fruiting, without crown competition between trees, mostly dispersed by gravity and seed dissemination by secondary dispersal agents such as birds. The fruiting season is from October to March, with a peak in November, coinciding with the rainfall peak. Ecological implications of these findings and alternative arguments to explain the high genetic diversity at regional landscape are discussed.
Key words - dioecy, floral visitors, flowering, fruiting, secondary forest

RESUMO – (Biologia reprodutiva de *Protium spruceanum* (Benth.) Engler. (Burseraceae), uma espécie arbórea dióica e dominante em corredores de vegetação, sudeste do Brasil). *Protium spruceanum* é uma árvore de dossel (com altura de até 20 m), ocorrendo comumente nas florestas Amazônica, Atlântica e nos cerrados exclusivamente no interior das matas ciliares. A fenologia e biologia reprodutiva foram avaliadas mediante observações quinzenais pelo período de um ano, em corredores de vegetação secundária em Lavras, sul de Minas Gerais. As análises da biologia floral compreenderam a viabilidade polínica, produção de néctar, receptividade estigmática, crescimento do tubo polínico, visitantes florais e taxas de visitas. As flores são pequenas (0,3–0,4 cm

de diâmetro), de cor amarelas claras, funcionalmente unissexuais e dispostas em densas inflorescências (45 flores). A atividade reprodutiva ocorreu durante a estação de maior precipitação. *P. spruceanum* apresentou floração anual e massiva com picos de floração rápidos e sincrônicos entre os dois sexos, entre Setembro e Novembro. As flores estaminadas produzem grande quantidade de pólens viáveis (90,6%) e relativamente de néctar (4,5 μL). Flores pistiladas oferecem apenas néctar para os visitantes (4,0 μL). Os polinizadores efetivos são *Apis mellifera* e *Trigona* sp. (Hymenoptera, Apidae). O tubo polínico de flores de polinização cruzada foi observado na base do estilete e ovários após 48 h da polinização. *P. spruceanum* apresenta alta taxa de frutificação, sem competição entre copas, possui dispersão barocórica e disseminação das sementes por agentes secundários, como pássaros. A frutificação ocorre entre Outubro e Março, com pico em Novembro, coincidindo com o período de maior precipitação. São discutidas as implicações ecológicas dos resultados e os argumentos para explicar a alta diversidade genética da espécie na paisagem local.
Palavras-chave - dioicia, floração, floresta secundária, frutificação, visitantes florais

1 Introduction

Many members of the Burseraceae family which is well-known for sources of gums and resins (Machado et al., 2003; Sunnichan et al., 2005), are dioecious and produce small flowers. Information on their reproductive biology however is available for only a few species of this family (Gupta et al., 1996; Farwig et al., 2004; Sunnichan et al., 2005; Voigt et al., 2005). *Protium* Burm. f. is a genus of approximately 146 species, occurring mainly in neotropical regions. The primary centre of diversity is in Amazon rainforest, where 73 species occur, of which 42 are endemic to the region (Daly, 1992). *Protium spruceanum* (Benth.) Engler, locally known as "breu", is a large canopy tree (up to 20 m tall), found in the Amazon and the Atlantic rainforests and on the cerrado riverbank woodland (Oliveira Filho & Ratter, 1995). The composition of the essential oils from leaves, thin branches and resin of *P. spruceanum* has been investigated (Machado et al., 2003). The resin of *P. spruceanum* is used to medicate for stomach ache and to relieve headaches and toothache; the leaves are used against fever (Milliken et al., 1986).

The Brazilian Atlantic forest, in the southern part of the Minas Gerais state, has been seriously exploited since European occupation two centuries ago resulting in the fragmentation and isolation of the population. At the same time, ditches to divide rural properties (*ca.* 4–6 m wide) were constructed by slaves, resulting in the vegetation corridors, that is, second-growth colonization by native tree species that connect small fragments of undisturbed primary forest (Vieira & Carvalho, 2008a). In our previous research, a study of *P. spruceanum* throughout the fragments of undisturbed Atlantic primary forest and corridors of secondary forest revealed that the species exhibited high levels of genetic diversity. It was hypothesized that this high diversity was associated with the reproductive system of this species (Vieira & Carvalho, 2008a). However, no data about reproductive biology of this species were available, so further work was needed to corroborate for this hypothesis. Correlations between genetic variation and reproductive data have been shown to be linked (Crawford & Elisens, 2006; Mateu-Andrés & Paco, 2006). For this, we investigated the reproductive biology and phenology of *P. spruceanum*, in the Atlantic forest in Lavras, southern Minas Gerais, Brazil.

2 Materials and methods

Study site – This study was conducted in vegetation corridors located in the city of Lavras, in southern Minas Gerais State, Brazil (21°17'33"S and 44°59'15"W, 21º18'11"S and 44°59'18"W). The region is characterized by a Köppen type Cwa climate, with a rainy summer and a dry winter season, the mean annual rainfall is approximately 1500 mm and the mean annual temperature is approximately 20° C. The current landscape comprises a number of primary forest fragments and a matrix of planted pastures and vegetation corridors of secondary forest (Vieira & Carvalho, 2008a). The area of the fragments and length of the vegetation corridors vary from 1 ha to 12 ha and 460 m to 1000 m, respectively. Fragments and vegetation corridors are similar in terms of floristic composition, but corridors are denser, have larger basal areas and trees are concentrated in the upper diameter classes and lower height classes. In the corridors studied, *Protium spruceanum*, along with companion tree species such as *Tapirira guianensis* Aublet (Anacardiaceae), *Copaifera langsdorffii* Desf. (Fabaceae) and *Ocotea pulchella* Mart. (Lauraceae) are the most abundant species of the mass-flowering/insect-pollinated and bird-dispersed tree species.

The absolute density of *P. spruceanum* in these corridors was estimated as 135 trees ha^{-1} (Castro, 2004).

Phenological observations and nectar production – Phenological observations for all reproductive trees were made fortnightly from November 2005 to December 2006, recording initial and final phenological periods. Absence or presence of buds, flowers and fruits were recorded in four vegetation corridors (*ca.* 2650 m length). For instance, flowering (anthesis) and fruiting (green and ripe fruit) were recorded as the presence of one or more open flowers or one or more fruit, respectively. Rainfall and temperature data were available during phenological observations from the Estação Meteorológica da Universidade Federal de Lavras (approximately 8 km north of study site). The number of flowers per inflorescence was estimated by counting the number of flowers in seven trees (2–4 inflorescences per tree). Nectar volume (in microliters) and sugar concentration (brix measurements) were measured on 61 bagged flowers from six trees, using graduated micro-capillaries and a hand refractometer. The volume and concentration of the accumulated nectar were estimated at approximately 08:00 h, when anthesis had started.

Pollen viability, stigmatic receptivity and pollen tube growth – Pollen viability was estimated in 13 flowers, using the aceto-carmine staining technique (Radford et al., 1974), under a microscope. Stigmatic receptivity was determined through the peroxidase activity technique (Kearns & Inouye, 1993), between 08:00 and 15:00 h, in both sexual morphs (N = 69, from six individuals). Pollinated flowers resulting from cross-pollinations were collected, and the styles removed and fixed with FAA for 24 hours. Following this, they was transferred to ethanol (70%) and then stored it at 8 °C. Pollen tube growth was investigated in pistils fixed 24 and 48 h after hand pollination. The styles were softened for 30 min in NaOH 6M in a water bath (60° C) and then rinsed in water. We stained the styles with 0.1% aniline blue for 4 h, squashed them under a cover-slip, and observed them under a fluorescence microscope (Martin, 1959).

Floral visitors – Floral visitor activity was recorded during two days of field observations, usually from 07:00 to 16:00 h. The resource gathering and movements regarding contact with stigma were recorded for all visitors. Three observers working separately enabled approximately 54 hours of observations to be recorded. Once observations were complete, we captured at least two individuals of each visitor morphospecies for identification. The frequency

curves of floral visitor was compared by Kolmogorov-Smirnov's nonparametric test (Sokal & Rohlf, 1995).

3 Results

Protium spruceanum presented massive annual flowering with quick and well synchronized blooming peaks of both male and female individuals, between September and November (Table 1). Flowering commenced with the beginning of the rains (Figure 1). Floral buds started to develop in July, in the transition period from dry to wet season. Overall reproductive activity was greatest in the rainy season and lower or absent in the dry season. The species is cryptically dioecious, with apparently perfect flowers possessing either rudimentary gynoecium lacking ovules (male plant) or vestigial anthers with no pollen (female plant). Anthesis started in the early morning and flower opening occurred during the day for flowers of both sexes. Male flower offered pollen and nectar while female flowers produced only nectar for flower visitors. Staminate flowers supplied a large quantity of viable pollen (90.6% ± 1.2 s.e, N = 13 flowers) coinciding with the reproductive phase of the stigma of the pistillate flowers that manifested immediately after flowering and extended throughout the day. Staminate and pistillate flowers produced relatively abundant nectar (4.5 and 4.0 µL, respectively), with an average of 28.3% and 32% concentration in sucrose equivalents, respectively. The trees had dense inflorescences (median = 45 flowers, range = 15–87, N = 22).

The flowers of *P. spruceanum* were visited by a low total number of insect species, including bees, wasps, flies and small ants, though only bees were effective pollinators. The bees *Apis mellifera* and *Trigona* sp. (Hymenoptera, Apidae) were the most frequent and abundant visitor species, probably due to the high number of flowers/inflorescence of *P. spruceanum* in the corridors of secondary forest. *Trigona* sp. individuals remained for several minutes foraging on a single flower. They remained for most of time in a single plant, compared to *A. mellifera*, which restricted their visits to less than a minute per flower and foraged fewer flowers per plant. Agonistic encounters interespecific were not common. The flowers of *P. spruceanum* were visited mainly between 09:00-12:00 h when most of the floral visitors contacted anthers and stigma. The difference in the frequency of visits between *Apis mellifera* and *Trigona* sp. was not significant ($P > 0.05$, Kolmogorov-Smirnov's test) (Figure 2).

Cross-pollen on pistils germinates and tubes grow down the style for up to 24 h (Figure 3A). Pollen tubes of cross-pollinated flowers were observed at the base of the style entering the ovaries 48 h after pollination (Figure 3B). Cross-pollinated flowers allowed normal pollen germination and pollen tube growth, and resulted in fruit and seed-set. This species is characterized by high rate of fruiting, without crown competition between trees, mostly dispersed by gravity and seed dissemination by secondary dispersal agents such as birds. The medium-sized, bird-dispersed seeds (< 500 mg fresh weight) are produced in reddish berries in the canopy of adult trees and are dispersed from October to March, with a peak in November, coincidently when the rainfall peaks. The fruits have an outer covering that splits in half when mature, exposing between one and three grey seeds, enveloped by a fleshy white aril that is dispersed by birds.

4 Discussion

The phenological pattern for our sample showed a trend toward a concentration of flowering and fruiting in the early wet seasons at the warmest time of the year. This is in accordance with the generally findings in the tropics, particularly where there is climatic seasonality (Schaik et al., 1993). Likewise, in Atlantic forest the flowering, in general, is highly seasonal and concentrated at the beginning of the wetter season (Morellato et al., 2000). This might be a consequence of marked irregularity in rainfall distribution and amount, which could be expected to lead to irregularity in flowering for certain species. The development of ripe fruits at the beginning of the warm and wet season is likely to be adaptive, so that germination and establishment can take place while water is plentiful (Schaik et al., 1993; Vieira & Carvalho, 2008b). Indeed, visual observations in the field indicate that germination of seeds and *P. spruceanum* seedling emergence occurs in the rainy season.

Our study contributes to a growing database on reproductive biology of the mass-fruiting, insect-pollinated and secondary bird-dispersed tropical trees, and of the species of Burseraceae, for which information on the reproductive biology is available for only a few species of this family (Gupta et al., 1996; Farwig et al., 2004; Sunnichan et al., 2005; Voigt et al., 2005). Our observations show that *P. spruceanum* presents massive flowering in vegetation corridors of secondary forest. Mass flowering represents the most extreme example of flowering synchrony at both the individual and population level (Frankie et al.,

1974; Gentry, 1978). Flowering synchrony can influence the levels of gene flow and differentiation among populations (Soliva & Widmer, 1999) depending on the effects of other organisms such as pollinators and seed dispersers (Domínguez et al., 2005). High levels of synchrony at both the individual and population level may increase the number of pollinators attracted to flowers and the rates of outcrossing (Augspurger, 1981), resulting in extensive pollen dispersal and gene flow (Byrne et al., 2007). Indeed, low genetic differentiation among primary forest fragments and vegetation corridors of secondary forest was observed in a previous allozyme study of *P. spruceanum* (Vieira & Carvalho, 2008a). Spatial patterns of genetic variation within populations depend primarily on the patterns and distance of pollen and seed dispersal and these results have often been interpreted as the consequence of intense gene flow (Doligez & Joly, 1997; Streiff et al., 1998).

Outcrossing rate increases with plant density (Calvo & Horvitz, 1990; Murawski & Hamrick, 1991; Treuren et al., 1993) and the difference in the local abundance of flowering plants (plant's floral neighbourhood) (Feinsinger et al., 1986) is thought to have a major influence on individual reproductive success (Feinsinger et al., 1991; Agren, 1996). The dense floral neighbourhoods are visited by pollinators more frequently, and as a result have increased rates of pollen deposition and fruit production (Calvo & Horvitz, 1990). Two of the most observed visitors of dense inflorescences of *P. spruceanum* are *Apis mellifera* and *Trigona* sp. (Apidae), and the opportunistic behavior of such family has been reported in many habitats and associated with plant density and clusters (Ramalho, 1990). Moreover, the dense inflorescences of the species (45 flowers/inflorescences) can attract more pollinators and, consequently, enhance fruit production. In fact, the high adult density (135 trees ha^{-1}), associated to the dioecious nature of the plants should favour the increased genetic diversity of *P. spruceanum* (Vieira & Carvalho, 2008a). In a recent review about outcrossing and pollen-mediated gene flow in neotropical trees, Ward et al. (2005) found no mating system studies (e.g. paternity analysis) for species of Burseraceae. However, Dunphy & Hamrick (2007) found long-distance pollen movement, combined with almost total outcrossing, which was likely to be responsible for the low levels of population divergence and the relatively high genetic diversity for the neotropical tree *Bursera simaruba* (Burseraceae).

Apis mellifera and *Trigona* sp. bees were considered efficient pollinators due their abundance at the flowers and also because they could easily contact anthers and stigmas. However, due to its high frequency of visits and its

efficiency as a pollinator, we consider *Apis mellifera* to be responsible for most of the natural pollination. This is consistent with other studies with bees being the most important pollinators for other tropical tree species of the family Burseraceae (Bawa, 1990; Farwig et al., 2004; Voigt et al., 2005). *Apis mellifera* is the most widely distributed exotic pollinator in the world (Kearns et al., 1998). In the Western Hemisphere, where African honeybees were introduced, they can be important competitors of native pollinators (Roubik, 1983; Huryn, 1997; Carmo et al., 2004). On the other hand, exotic honeybees sometimes have neutral or even beneficial effects on the pollination of native plants (Gross, 2001; Dick et al., 2003; Suzuki, 2003), because of their high density, social organization and propensity for agricultural landscapes (Dick et al., 2003). High temperatures around midday could result in high insect activity, as observed in this study, and this could lead to high visiting rates (Arroyo et al., 1985).

Male and female trees can differ in their attractiveness to pollinators; staminate flowers provide pollen and mostly nectar, whereas pistillate flowers often have only nectar (Bawa, 1990). Consequently, the main advantage of dioecy may be avoidance of selfing. This might result in high fruit set in comparison with monoecious and hermaphroditic plant species (73.8% vs. 42.1%) (Sutherland & Delph, 1984). Indeed, vegetation corridors presented high gene diversity, historical gene flow with the fragments and absence of inbreeding (Vieira & Carvalho, 2008a). Nevertheless, further studies of sex ratio variation, spatial distribution and parentage analysis (Dunphy & Hamrick, 2007; Pavón & Ramírez, 2008) across the vegetation corridors are necessary to provide a clear picture of the contribution of seed and pollen to the overall contemporary gene immigration. These ideas are now being researched (Vieira et al., 2008). The comparison between historical estimates of gene flow, using variance in allelic frequencies, and contemporary estimates of gene flow, using parentage assignment or reproductive biology data, is expected to provide insights into ecological and evolutionary processes at regional landscape within and among populations.

Acknowledgments – The authors acknowledge Grazielle S. Teodoro for help during field work. Dra. Giovana A. Torres and Dra. Roselaine C. Pereira for their contributions in pollen tube growth analysis. Dr. Geoff Carr-UK for his suggestions on the manuscript. The Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Conselho Nacional de Pesquisa (CNPq) are also ackowledged for providing a Dr. fellowship for F.A. Vieira, a research fellowship for Dr. Dulcinéia de Carvalho and research grant.

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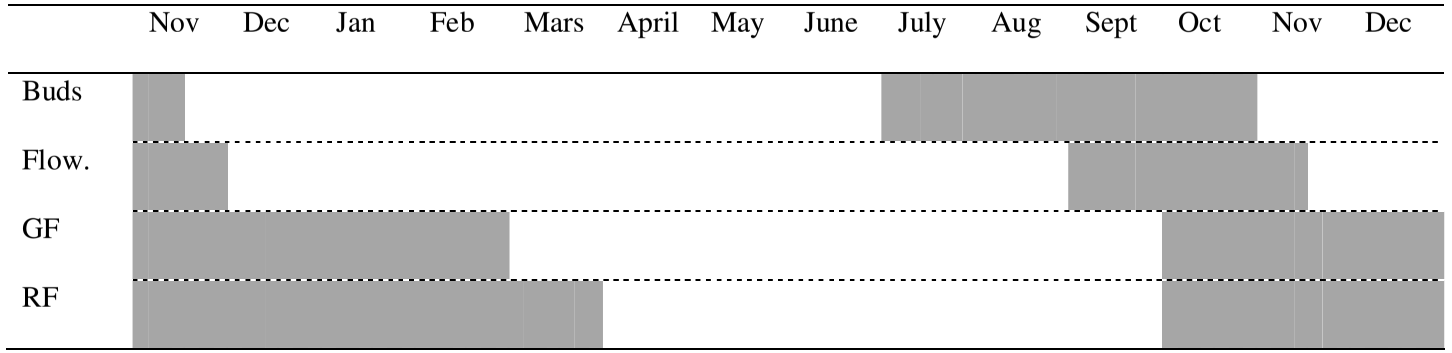
Table 1. Fortnightly phenological events of individuals of *Protium spruceanum* records from November 2005 to December 2006, in vegetation corridors, Minas Gerais, Brazil. Flow, flowering; GF, green fruit; RF, ripe fruit.



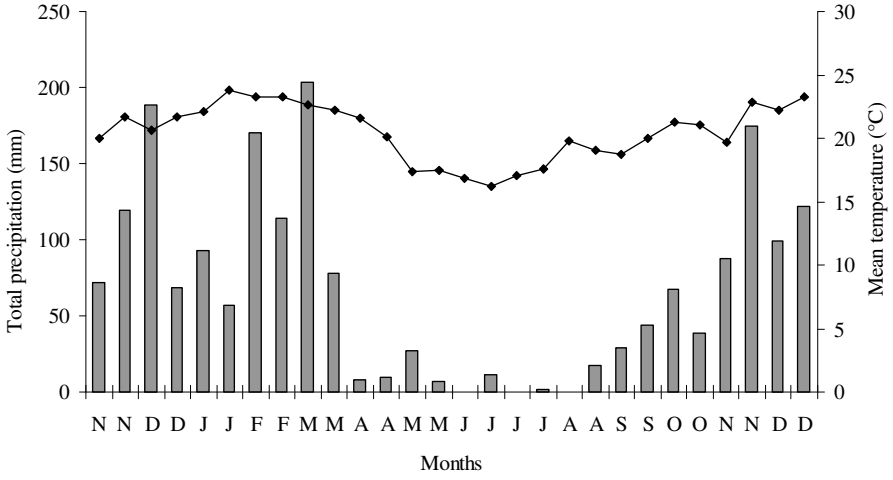


Figure 1. Meteorological data for the studied site in Lavras, Minas Gerais State from November 2005 to December 2006 (precipitation = bars, temperature = lines).

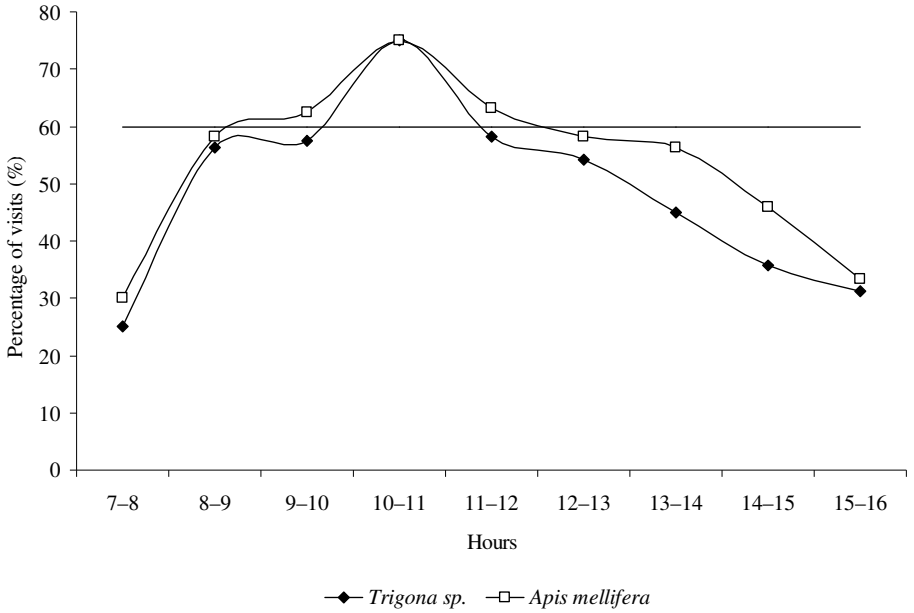


Figure 2. Frequency of visits of *Apis mellifera* and *Trigona* sp. on flowers of *Protium spruceanum* during all the monitored hours. The line in the percentage 60 was used to detach the periods with high floral visitors (> 60%).

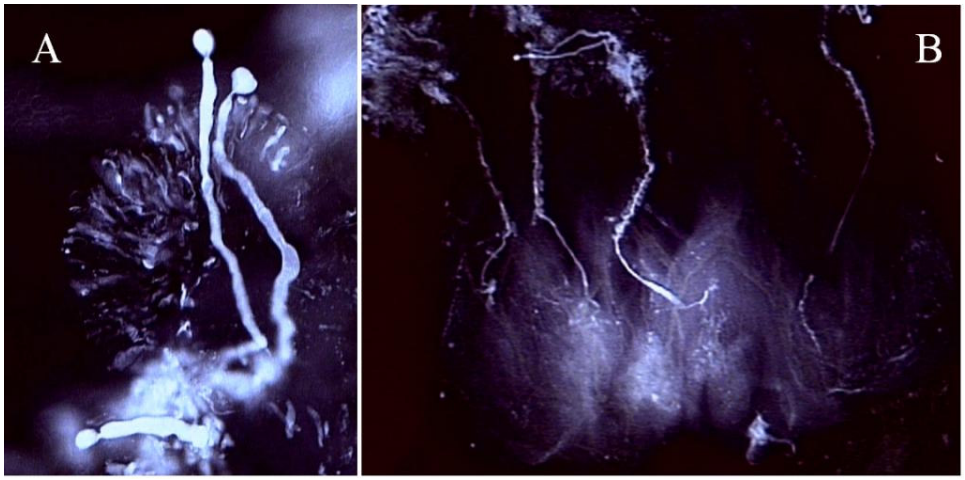


Figure 3. Fluorescence photomicrography of pollen tube growth of *Protium spruceanum* after pollinations. Cross-pollen in the stigma germinates (A). Pollen tubes of cross-pollinated flowers at the base of the style 48 h after pollination (B).

ARTIGO 4

Demographic Fine-scale Genetic Structure of *Protium spruceanum* (Burseraceae), a Dioecious-dominant Neotropical Tree, on Brazilian Atlantic Population

(Preparado de acordo com as normas da revista *Annals of Botany*)

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Running title: **Spatial Genetic and Demographic Structure in a Neotropical Tree**

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• *Background and Aims* The knowledge of demography in the analysis of fine-scale genetic structure (SGS) provides critical information about the mechanisms responsible for the observed patterns. We present a case study of the relationship between fragmentation, SGS and age structure in an insect-pollinated, mass-fruiting and secondary bird-dispersed tree, as inferred by variation at allozyme loci.
• *Methods* Using ten polymorphic loci, we investigated spatial and temporal patterns of genetic structure within a 40 m x 60 m area in small (1.0 ha) undisturbed Atlantic primary forest to infer processes shaping the distribution of genetic diversity. Four categories of plants (N = 345), from seedlings to adults according to diameter classes, were analyzed.
• *Key Results* The results showed a high average population level of gene diversity (H_e = 0.438), but genetic diversity parameters did not vary significantly among cohorts. The spatial distribution pattern analysis showed significant levels of aggregation at small and medium diameter-class and random distribution at the high diameter-class, probably due to compensatory mortality during recruitment and survival under competitive thinning process. There was a strong link between demographic and genetic spatial structures at small distances (less than 10 m) which is likely to be the consequence of restricted seed dispersal. The magnitude of SGS was observed to be decreasing from smaller- to larger-diameter classes. We inferred that limited seed dispersal and subsequent random loss of individuals from the family patches are responsible for the observed changes in fine-scale SGS across different demographic classes.
• *Conclusions* The spatial genetic and demographic structures suggest efficient and rapid colonization dynamics in small undisturbed primary fragment. Moreover, this study shows how genetic diversity can be maintained due to natural plant recruitment post-fragmentation, a positive aspect for *in situ* conservation.

Key words: *Protium spruceanum*, allozymes, cohorts, genetic diversity, spatial genetic structure, spatial pattern.

1 INTRODUCTION

The knowledge of demography in the analysis of genetic structure provides critical information about the mechanisms responsible for the observed genetic structure (Ng et al., 2004; Hardesty et al., 2005; Chung et al., 2006). Indeed, the patterns of spatial genetic structure (SGS) within local plant populations are the result of several genetic and demographic processes acting at specific temporal and spatial scales (Jones et al., 2006; Jones & Hubbell, 2006). SGS is influenced by biological aspects of a tree species, strongly dependent on life stage, particularly in species with long life cycles such as trees. Studies that have examined transitions among different life stages in plants have revealed two diametrically opposed sets of results: an increase in relatedness among neighboring individuals from seedling to adult stages (Tonsor et al., 1993; Kalisz et al., 2001; Latouche-Halle et al., 2003; Jones & Hubbell, 2006), and decreases in relatedness from juveniles to reproductive adults (Hamrick et al., 1993; Epperson & Alvarez Buylla, 1997; Ng et al., 2004; Hardesty et al., 2005). There are several possible explanations for these patterns, but generally speculated that historical factors, local adaptation, nonequilibrium population dynamics, limited dispersal and overlapping generations can lead to an increase in SGS towards the adult stages (Kalisz et al., 2001; Latouche-Halle et al., 2003; Jones & Hubbell, 2006). Limited dispersal near the parent plant and low density of reproductive adults can explain the decay of genetic structure across size classes (Hamrick et al., 1993; Epperson & Alvarez Buylla, 1997).

Analysis of SGS over the life cycle can infer the roles of these demographic and genetic processes in arise and maintenance of the within population genetic structure (Kalisz et al., 2001; Chung et al., 2003). Nonetheless, few studies in tropical forest have examined the different life-history stages at both demographic spatial distribution and fine-scale SGS within populations; and associated causal processes are not well understood (Latouche-Halle et al., 2003; Ng et al., 2004; Hardesty et al., 2005; Silva et al., 2008). Although there have been few such studies, to our knowledge, only the study of Conte et al. (2003) had examined the changes in genetic structure through life history stages in the threatened Brazilian Atlantic forest, but the spatial distribution and fine-scale SGS pattern of cohorts were not evaluated. These authors investigated the genetic diversity and recruitment of plants of heart-of-palm tree (*Euterpe edulis*) and the results showed the maintenance of high

diversity levels, as well as the significant increase in the heterozygote frequency towards the adult stages.

In our previous research, allozyme study of *Protium spruceanum* throughout the fragments of undisturbed Atlantic primary forest and corridors of secondary forest revealed that the species exhibited high levels of genetic diversity and most of the diversity was partitioned within populations (Vieira & Carvalho, 2008). Moreover, this diversity is not structured within populations, because the genetic differentiation among fragments and SGS of large trees within of the fragments were low. However, given the longevity of most tree species, the study of the next generations will be required to provide a clear picture of the genetic outcome of the studied populations. *P. spruceanum* is a large canopy tree (up to 20 m tall), dioecious insect-pollinated and shade-tolerant, since the recruitment of seedlings can occur under large trees. This species is characterized by high density, without crown competition between trees and high rate of fruiting, mostly dispersed by gravity and seed dissemination by secondary dispersal agents such as birds. Thus, if seed dispersal by secondary agents is restricted and pollen movement is limited, our hypothesis is the increase of genetic relatedness and spatial aggregates among young individuals in close proximity. In addition, it would be expected that both demographic and genetic spatial structures disappears among neighboring individuals across life-history stages.

The demographic and fine-scale SGS between the juveniles and adult cohorts were compared in order to identify the processes modifying diversity during the lifetime of the trees. The specific objectives of our study were: (1) to describe the genetic diversity of a mapped individuals of tree species *P. spruceanum* within a fragment using allozyme; (2) to compare the genetic diversity parameters among four life stages; and (3) to evaluate the levels of spatial aggregation and spatial genetic structure of the cohorts at fine-scale.

2 MATERIALS AND METHODS

Sampled site

The fragment studied is located in the region of Lavras, South of Minas Gerais State, Brazil. The forest fragment (*c*. 1.0 ha) is located at 21º17’52’’S and 44º59’13’’W, and at 973 m of altitude. The population studied have rapidly declined because of habitat fragmentation caused by anthropogenic disturbance over the last 200 years, corroborates by detection of recent bottlenecks (Vieira &

Carvalho, 2008). These vegetal formations were seriously plundered since European occupation goes back to colonial times (two centuries ago), resulting in the fragmentation and isolation of these populations at a particularly rapid pace. The species occurs in a large part of the fragment, which may be determined by the almost permanently flooded soil. *P. spruceanum* is the most abundant tree species in the fragments. All individuals found in the census within a 24 adjacent (100 m^2) sample plots, 40 m x 60 m area, were mapped and these variables were translated into *x-y* coordinates between all pairs of individuals **[Supplementary Information]**. Plants frequency distributions into classes of diameter were prepared using class intervals with exponentially increasing ranges to make up for the normally steep decrease in tree density toward larger diameters, as suggested by Oliveira Filho et al. (2001) (Fig. 1).

In total, the population sampled for the genetic study included 345 plants (mean of 68.4 % of the census) classified according to diameter at breast height (d.b.h.) or at soil height (d.s.h.) into four diameter classes (Fig. 1 and Table 1). Samples were selected within all 24 sample plots (Fig 2.). Tissue of SMA cohort was collected only from healthy plants in which there was sufficient leaf material such that removal of tissue would be unlikely to cause mortality. Live tissue was transported on ice to the laboratory and stored at –80 °C until enzyme extraction.

Enzyme extraction and electrophoresis

Small pieces of leaf tissue were crushed in 1 mL of the extraction buffer as described in Vieira & Carvalho (2008). Discontinuous vertical electrophoresis was performed in a polyacrylamide gel (10 %) and carried out at 4 °C over 3 h (constant current of 80 mA and voltage of 300 V). Nine enzymatic systems were used: acid phosphatase (E.C.3.1.3.2, locus *Acp*), alcohol dehydrogenase (E.C.1.1.1.1, locus *Adh*), glucose dehydrogenase (E.C.1.1.1.47, locus *Gdh*), β-galactose dehydrogenase (E.C.1.1.1.48, locus *Gldh*), glutamate dehydrogenase (E.C.1.4.1.3, locus *Gtdh*), malate dehydrogenase (E.C.1.1.1.37, locus *Mdh2*), peroxidase (E.C.1.11.1.7, loci *Per1* and *Per2*), sorbitol dehydrogenase (E.C.1.1.1.14, locus *Sdh*) and shikimate dehydrogenase (E.C.1.1.1.25, locus *Skdh*). Staining protocols and the genetic basis of allozymes banding patterns were inferred from segregation patterns with reference to typical subunit structure and conceptual methods described in Wendel & Weeden (1989).

Putative loci and alleles were designated sequentially. The one with the most anodally migrating allozyme or alleles was designated as *1* and the next as *2*.

Genetic diversity

Within each cohort, intrapopulation genetic diversity was analyzed by percent of polymorphic loci (P_L; 0.95 criterion), mean number of alleles per locus (A), observed heterozygosity (H_o), Nei's gene diversity (H_e) and the fixation index estimates (f). Standard errors for H_o and H_e were calculated over loci. Significant negative f was tested using 1000 randomizations for each locus. Departures from Hardy–Weinberg (H–W) equilibrium at each locus were tested in each cohort using the Fisher exact tests. These calculations were performed using BIOSYS–1 (Swofford & Selander, 1989) and GDA 1.1 (Lewis & Zaykin, 2001) programs. Comparisons among cohorts according to these parameters (H_e and f), were performed using the program FSTAT 2.9.3.2 (Goudet, 2002), through the G-based exact test proposed by Goudet et al. (1996). Potential linkage disequilibrium between allozyme loci was tested in cohorts using POPGENE version 1.31 (Yeh et al., 1999) with the significance level corrected for multiple comparisons (Rice, 1989).

Genetic differentiation

We calculated pairwise differentiation between cohorts using F_{ST} statistics (Weir & Cockerham, 1984). A G-test, based on 6000 permutations of genotypes among samples, was performed to test for cohorts differentiations at allozymes loci (Goudet et al., 1996). Pairwise tests of differentiation were performed using the G-statistic by randomization of multilocus genotypes for allozymes. We adjusted the significance level for multiple pairwise comparisons using a sequential Bonferroni correction. These calculations were performed using the program FSTAT.

Demographic data analysis

The spatial distribution of individual's census were tested for clumping using univariate second-order spatial pattern analysis, based on Ripley's (1976) K-function. This method considers all individuals within a given radius t of the focal individual. We used the modified L-function, defined by Besag & Diggle (1977) as: $L(t) = \sqrt{k(t) / \pi} - t$. This L-function has a more stable variance than

the *K*-function, and is easier to interpret: $L(t) = 0$ under complete spatial randomness (CSR); $L(t) < 0$ indicates inhibition, i.e. there are fewer neighbors within a distance t of an arbitrary point of the pattern than expected under CSR, so that the pattern tends to be regular; $L(t) > 0$ indicates aggregation, i.e. there are more neighbors within a distance t of an arbitrary point of the pattern than expected under CSR, so that the pattern tends to be clustered. Weighted edge corrections, based on those of Goreaud & Pelissier (1999) were calculated. The 99 % confidence intervals for the statistic were estimated by performing a Monte Carlo procedure with 499 replicates for $\alpha = 0.01$, and the sample statistic was compared with this envelope. These calculations were analyzed with SpPack version 1.38 (Perry, 2004).

Spatial genetic structure

Under isolation by distance, neighborhood size was estimated (using only adult trees) as $Nb = 4\pi d_e s^2$, where d_e is the effective population density and 's' is the mean square distance of gene dispersal, following Fenster et al. (2003). Cohorts' SGS was further analyzed using Nason's kinship coefficient (or coancestry) (Loiselle et al., 1995). This coefficient can estimate between pairs of mapped individuals x and y a ratio of differences of probabilities of identity-in-state between homologous genes (Rousset, 2002). For fine-scale SGS analysis, distance class intervals between individuals were determined by testing to get rule of thumb for each distance interval as suggested by Hardy & Vekemans (2002). To test for significant deviations from random SGS, observed values for each distance class were compared to the 95 % confidence interval derived from 1000 permutations. The extent of SGS was estimated using the *Sp* statistic following Vekemans & Hardy (2004). The *Sp* statistic here is used as a simple measure to allow for comparisons among cohorts, not as an estimate of the variance in gene dispersal distances. *Sp* was quantified by $Sp = -b_{log}/(1 - F_{(10,m)})$, where b_{log} is the regression slope and $F_{(10,m)}$ is the mean kinship coefficient between individuals belonging to the first distance interval (0–10 m). The b_{log} standard errors were obtained by jack-knifing over loci. These calculations were performed using the program SPAGeDi 1.2g (Hardy & Vekemans, 2002).

3 RESULTS

Genetic diversity

P. spruceanum shows a high level of genetic diversity across all diameter classes observed (Table 1). The nine enzyme systems used showed ten loci that could be interpreted and 20 alleles **[Supplementary Information]**. No exclusive alleles were detected by analysis of the allele frequencies of the 10 polymorphic loci in the four cohorts studied. The mean percentage of polymorphic loci within local cohorts was 100.0 % and segregating two alleles per locus. The relationship between the observed (H_o) and expected (H_e) mean heterozygosities resulted in a negative fixation index (f) in all the cohorts analyzed, indicating a high proportion of heterozygotes and the absence of inbreeding (Table 1). Out of 40 tests for H–W expected genotypic frequencies (4 cohorts by 10 loci) three showed significant ($P < 0.05$) departures from equilibrium, after sequential Bonferroni correction. Locus *Sdh* in SMA and loci *Gldh* and *Per2* in MED2 cohorts were affected, but this low number of significant tests suggests no overall departure from H–W equilibrium. Twenty-seven potential linkage disequilibrium tests, out of 760, were significant which is slightly higher than what would be expected by chance at the 0.05 level, after Bonferroni correction. Twenty-one tests were significant in the SMA cohort and six in the MED2 cohort. Tests for linkage disequilibrium provided no evidence for physical linkage among loci in the MED1 and BIG cohorts.

The mean values of gene diversity and fixation index were not significantly different between the cohorts (Table 2). Less than 1.4 % of the genetic variation was partitioned among different diameter classes; the large majority of genetic variation was within diameter classes. Based on Goudet's *G*-test, differentiation between cohorts was overall no significant at the 5 % level (F_{ST} = 0.005; *C.I. confidence interval* = –0.001 to 0.016), but considering pairwise estimates, only young cohorts (MED2 vs. SMA) were significantly differentiated ($P < 0.01$), with a multilocus estimate of F_{ST} of 1.0 % (Table 2).

Spatial distribution

Our study plot contained 792 *P. spruceanum* individuals (Table 1), covering *c*. 24 % of the area. *P. spruceanum* showed a skewed size-class distribution with many small individuals and few large individuals (Fig. 1). The spatial distribution of plants was significantly different from random at SMA (t = 0–25 m) and MED2 (t = 2–11 m). For both cohorts, the *L*-function curves exhibited a divergence from the null-hypothesis of a random distribution of plants (Fig. 3); significant positive values suggested that plants are distributed in aggregates. Only MED2 cohort showed a spatial structure with clumped and random

patterns. The univariate analysis of the plants at MED1 and BIG cohorts showed no evidence of non-random spatial distribution. The magnitude of spatial aggregation was observed from high aggregation to random distribution as diameter class increases (SMA > MED2 > MED1/BIG).

Spatial genetic structure

Relative to 95 % confidence limits, the spatial autocorrelation analysis detected a significant SGS up to a radius of approximately 10 m in SMA and MED2 cohorts, based on the kinship coefficients. A continuous decrease in the autocorrelation values was detected with increasing distances in MED2 cohort, and from 65 m onward, it showed significant negative values, suggesting that nearby trees are genetically related and distant trees are not (Fig. 4). In the MED1 and BIG cohorts, coancestry values were within the range of 95 % confidence limits in all of the distance classes. The regression of pairwise kinship values on the logarithm of geographic distance had a significantly negative slope b_{log} ($P < 0.025$) indicative of SGS in the two young cohorts studied for *P. spruceanum*. Then, overall slopes (b_{log}) of correlograms of SMA and MED2 cohorts were significantly different from the null hypothesis of no SGS ($b_{log} = 0$): b_{log} of –0.009 (0.003 s.e.) for SMA cohort and b_{log} of –0.013 (0.013 s.e.) for MED2 cohort. The overall slope of the correlogram was not significantly negative in analysis of the MED1 and BIG cohorts. *Sp* statistics were 0.009, 0.013, 0.0004 and 0.011 in cohorts SMA, MED2, MED1 and BIG, respectively, with an average of 0.009±0.003. Neighborhood sizes were estimated (using only BIG cohort) as approximately 44 individuals.

4 DISCUSSION

Genetic diversity

Our findings show that cohorts of *P. spruceanum* in small primary forest fragment maintain high levels of allozyme diversity. Estimates of genetic diversity within the study population of *P. spruceanum* were not significantly different among cohorts. In tropical forest trees, generally genetic diversity parameters did not vary greatly according to cohorts (Hall et al., 1994; Conte et al., 2003; Kelly et al., 2004). Furthermore, our results suggest that this fragmentation event and recent bottlenecks, previously reported in this species and study site (Vieira & Carvalho, 2008) did not change the level of genetic

diversity between the old and young individuals. However, fragmentation of this population had occurred 200 years prior to the study, and is thus a recent event, relative to the lifespan of the species. Therefore, since trees that had probably been present prior to anthropogenic disturbances were sampled for this study (e.g. BIG cohort), the results may reveal current rates of differentiation between old individuals pre-fragmentation and young individuals after fragmentation. Few studies have examined the differences in the genetic diversity between cohorts pre and post-fragmentation in Brazilian Atlantic forest. Torezan et al. (2005) used random amplified polymorphic DNAs (RAPDs) to quantify genetic diversity within of populations of *Aspisdosperma polyneuron*, a long-lived, late-reproducing tropical tree, from adults (pre-fragmentation, >300 years old) and seedlings (post-fragmentation, <50 years old) and results showed a decrease of genetic polymorphism of post-fragmentation cohorts in small fragments in the Atlantic forest. Lowe et al. (2005) reviews studies in fragmented habitats and results are in line with theory, where inbreeding is often observed immediately following impact, but genetic diversity is lost slowly over subsequent generations, which for trees may take decades. However, these results indicated that tropical tree species have different responses to forest fragmentation, according particular life history characteristics (e.g. mating system, pollen and seed dispersal mechanisms) (White et al., 2002; Dick et al., 2003).

Fixation indices significantly deviated from zero in the negative direction in all cohorts. This can be seen as indicative of a primarily outcrossed mating system (Ge & Sun, 1999; Hoebee et al., 2006). Although the mean value of *f* across cohorts (–0.159) may indicate a general heterozygote excess over the panmictic expectation for *P. spruceanum*, the range of the fixation index in other neighbors forest fragments (from –0.250 to 0.078) (Vieira & Carvalho, 2008) suggests different selection levels. This could be due to random variation, heterogeneous pollen pool, positive assortative mating or phenological differences within and among trees (Shaw & Allard, 1982; El-Kassaby et al., 1984). Future studies could focus on the multilocus and single locus outcrossing rates to provide and a clear picture of the negative inbreeding coefficients.

If most of the seedlings (SMA) and juveniles (MED2) studied are the products of repeated long-distance dispersal of seeds by birds, from other nearby older populations, there may be no reason to expect genetic differentiation (F_{ST}) among these two classes. However, significant genetic differentiation (SMA vs. MED2; $F_{ST} = 0.010$, $P < 0.01$) was detected, suggesting temporally varying demographic processes and reproductive events might have operated in the study

population. For example, Chung et al. (2003) found highly significant genetic differentiation among age classes for an insect-pollinated and bird-dispersed tree and this was attributable to episodic demographic and reproductive events over generations. Moreover, evidence of a temporal Wahlund effect would be indicated by significant differentiation between age classes (Tonsor et al., 1993; Epperson & Chung, 2001). However, in *P. spruceanum* this effect may not explain the genetic differentiation among cohorts because the absence of the heterozygote deficit in the cohorts.

Spatial demographic and genetic structures

Spatial distribution pattern analysis showed that the majority of tropical tree species aggregated at various diameter classes (Hubbell, 1979; He et al., 1997; Okuda et al., 1997; Condit et al., 2000), normally from higher to lower aggregation or random distribution with age increase (e.g. *Alsesis blackiana* and *Platypodium elegans*) (Hamrick et al., 1993); *Cecropia obtusifolia* (Epperson & Alvarez-Buylla, 1997); *Camellia japonica* (Chung et al., 2003); *Shorea leprosula* (Ng et al., 2004). Similarly, in this study we found significant spatial aggregation in small- and medium-diameter classes; small-diameter class trees are generally more clumped than medium-diameter class trees. The possible mechanisms of clumping have been discussed from the viewpoint of seed dispersal (Plotkin et al., 2000), gap recruitment (Itoh et al., 1997; Plotkin et al., 2000), distance-dependent mortality (Itoh et al., 1997), density-dependent recruitment (Okuda et al., 1997), topography (Plotkin et al., 2000), pest effect (Harms et al., 2000), herbivores and plant diseases (Condit et al., 2000) and species density (Condit et al., 2000). In *P. spruceanum*, seed shadow due to limited seed dispersal determines basically the clumping of small-diameter class. If seeds fall beneath the maternal plants, individuals in younger age classes should exhibit a clumped spatial distribution. After seed dispersal, the compensatory mortality due to environmental heterogeneity or intra-specific competition on small-diameter class might lead to less clumping on medium-diameter class. Subsequently, the compensatory mortality due to micro-environmental selection, herbivores and plant diseases on medium-diameter class will increase the thinning process so that only few individuals will be able to survive and form the future adults, and this might cause the random distribution on large-diameter class (Ng et al., 2004). In addition, the distribution of the *P. spruceanum* plants across the different diameters classes followed the pattern of an inverted "*J*" curve, suggesting seedling bank behavior. Such

behavior in natural regeneration had been described previously for other species (Chung et al., 2003; Conte et al., 2003; Ruschel et al., 2006).

The level of aggregation detected here for SMA and MED2 cohorts (10 m or below), groups plants that are very similar genetically, as suggested by the spatial autocorrelation analysis (Fig. 4), and illustrated by the spatial distribution of individuals (Fig. 3). Hence, there is strong correspondence between demographic and fine-scale SGS in small distance classes. The congruence between the demographic and genetic structures is likely to be the consequence of restricted seed dispersal, meaning that the smaller size of aggregates would correspond to the dispersion distance from a single source (i.e. maternal tree, e.g. *Dicorynia guianensis*) (Latouche-Halle et al., 2003); *S. leprosula* (Ng et al., 2004); *Simarouba amara* (Hardesty et al., 2005). When pollen dispersal is random but seed dispersal is highly localized there will be no inbreeding and the spatial aggregations of siblings will result in significant fine-scale SGS (Hamrick & Nason, 1996).

The ecological and evolutionary processes (e.g. limited seed dispersal, competitive thinning, predation and environmental heterogeneity) that affect spatial distribution patterns can also be contributing factors to the observed fine-scale SGS, including also mating system and colonization history (Vekemans & Hardy, 2004; Jones et al., 2006). We found SGS in the young plants (SMA and MED2 cohorts), but this structure almost completely disappeared in the olds plants (MED1 and BIG cohorts). The decrease in magnitude of SGS from smaller- to larger-diameter classes observed is in accordance with other studies in forest tree species: *C. obtusifolia* (Epperson & Alvarez Buylla, 1997), *D. guianensis* (Caron et al., 2000), *S. leprosula* and *S. ovalis* (Ng et al., 2004), *Fagus crenata* (Asuka et al., 2004) and *S. amara* (Hardesty et al., 2005). This can be explained by the compensatory mortality and competitive thinning process during recruitment and selection in favour of different genotypes. The reduction pattern of SGS with the progress of the stage could occur when juveniles show genetically random mortality that occurs in a density-dependent way. In summary, under a high level of recruitment, SGS appears at the juvenile stage probably because of limited seed dispersal around maternal plants, and subsequent random loss of individuals from family patches is a plausible explanation for the loss of SGS from juvenile to reproductive stages. Skabo et al. (1998) argued that there would be a tendency for this to happen because the family grouping caused by limited seed dispersal would be reinforced each generation by limited pollen dispersal (Barbour et al., 2005) and biparental

inbreeding, but the tendency to build up genetic structure in the juvenile cohort would be countered by selection against the products of inbreeding as they mature (Hardner et al., 1998). Although no inbreeding was found in the SMA and MED2 cohorts, we paradoxically found relatedness at the shortest distance class. Fine-scale structure can develop when the variance in the seed dispersal is smaller than the variance in pollen dispersal (Kalisz et al., 2001). The clumped distribution of *Protium* juvenile cohorts, along with our relatedness in the SMA and MED2 juvenile cohorts, suggest that there is large overlap in the seed shadows of individual reproductive *Protium*. Thus, from the reduction in relatedness from the SMA to BIG stages, we expected to see a further decay of structure in larger diameter classes due to random thinning of individuals (Hamrick et al., 1993).

In the light of the *Sp*-based SGS review performed by Vekemans & Hardy (2004), SGS in *P. spruceanum* (*Sp* ~ 0.009) is consistent with other tree life form species (*Sp* = 0.010), other plant species with animal-dispersed seeds (*Sp* = 0.009) and to *P. spruceanum* adults trees at fine-scale SGS in the four fragments of undisturbed primary forest (*Sp* = 0.008) (F. A. Vieira et al., unpublished data, 2009). Our study contributes to a growing database on patterns of SGS for vertebrate-dispersed tropical tree species (Degen et al., 2001; Vekemans & Hardy, 2004; Hardy et al., 2006).

Conclusions

This study shows how genetic diversity can be maintained due to natural plant recruitment post-fragmentation. The absence of a clear differentiation among cohorts is thus consistent with the low differentiation observed among them and the absence of a reduction in gene diversity in young cohorts. The maintenance of this level of genetic diversity should allow this species to maintain its ability to adapt to novel environmental changes, mainly because of the possibility of the appearance of new recombinants in the next generations. Spatial genetic and demographic genetic structures suggest efficient and rapid colonization dynamics in small undisturbed primary fragment. Different factors could be responsible for a SGS in the young cohorts for *P. spruceanum*. In particular, this species is characterized by the high degree of fruiting, no crown competition between trees. In addition, good soil conditions might favour the important natural regeneration of siblings in the neighborhood of the mother trees, since seeds of *P. spruceanum* are mostly dispersed by gravity, and the restricted seed dissemination by secondary dispersal agents such as birds. If population

expansion resulted from local seed production and recruitment in the absence of local adaptational differentiation, then fine-scale SGS among seedlings and young individuals would be expected to be significant and stronger than among older individuals. As seedlings and younger individuals grow, competition among individuals within cohorts will be able to generate thinning, shifting from a clumped to random distribution with age. Thus, our study contributes to a growing database on patterns of demographic genetic structures for a mass-fruiting, insect-pollinated and secondary bird-dispersed tropical tree. Unlike previous studies though (Vieira & Carvalho, 2008), we consider not only the fragment forest but also the genetic diversity of recolonizing individuals. Although we examined only one population, it serves to validate our hypotheses and suggest further research. In addition, investigations on contemporary patterns of genetic and exhaustively sampling all individuals within the different populations is necessary. Our study will provide a useful reference to measure the levels of genetic diversity in other *P. spruceanum* populations. Further studies of direct methods (DNA-based) for estimating contemporary gene flow across the landscape are also necessary to provide a clear picture of the contribution of seed and pollen to the overall contemporary gene immigration (e.g. parentage analysis) (Sezen et al., 2005). Nevertheless, considering the practically irreversible fragmentation of populations and high genetic diversity found in small undisturbed primary forest, landscape management strategies should consider the protection of extant ones.

SUPPLEMENTARY INFORMATION

Supplementary information shows the distribution of individuals in the census and the allele frequencies of cohorts of *P. spruceanum* surveyed in this study.

ACKNOWLEDGEMENTS

We thank Flávio R. Gonçalvez, Rodrigo M.G. Melo and Rubens M. dos Santos for valuable field assistance. We also thank Eduardo van den Berg for all advices. The Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Conselho Nacional Pesquisa (CNPq) are also acknowledged for providing a Dr. fellowship for F.A. Vieira, a research fellowship for Dulcinéia de Carvalho and research grant.

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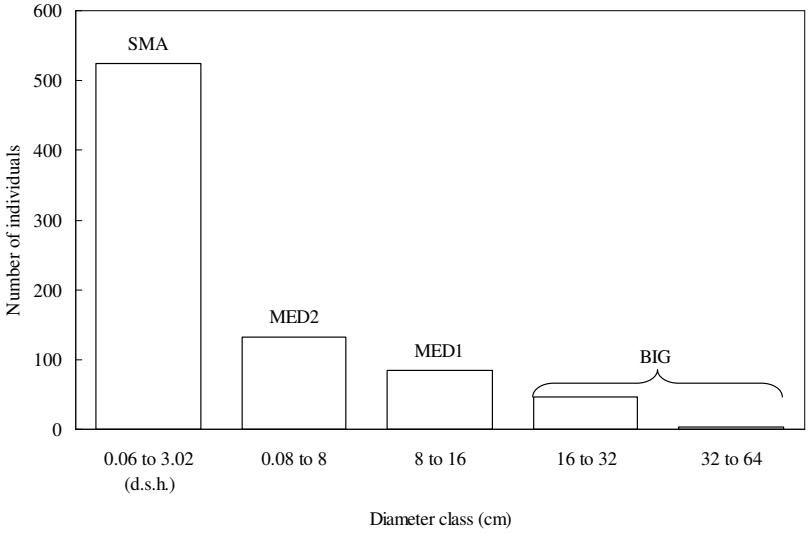
LEGENDS TO FIGURES

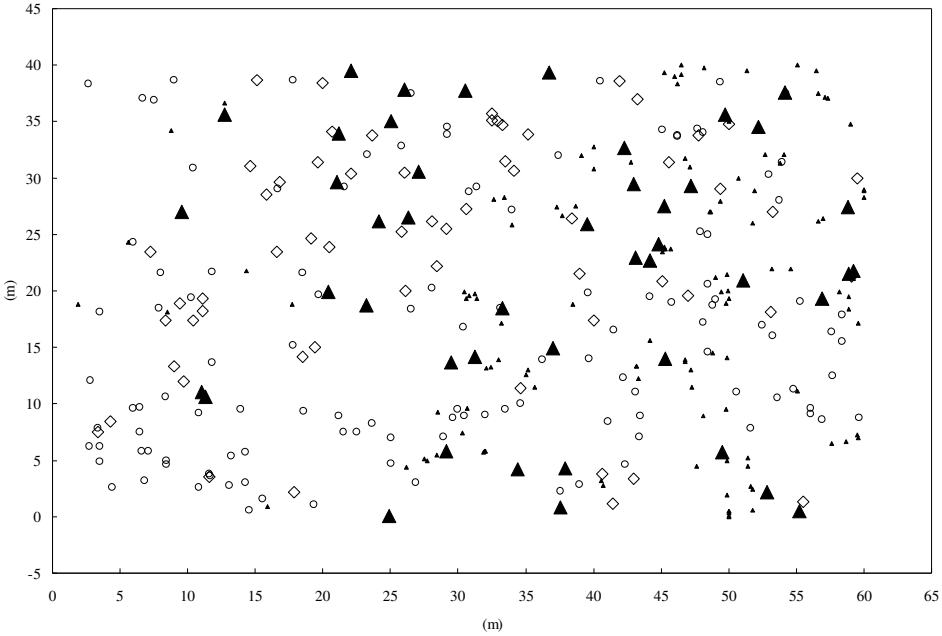
FIG. 1. *Distribution of the number of individuals of P. spruceanum per diameter classes in the 2400 m^2 of primary forest. Samples collected were classified according to diameter at breast height (d.b.h.) in MED2, MED1 and BIG cohorts or diameter at soil height (d.s.h.) in SMA cohort.*

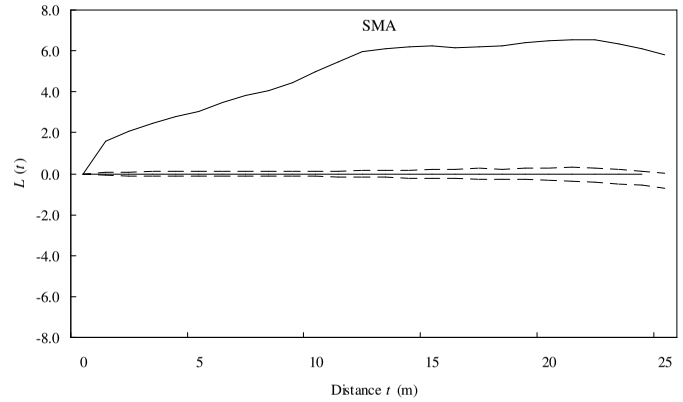
FIG. 2. *Distribution of the trees sampled in four cohorts for the analysis of the spatial genetic structure in P. spruceanum.* ▲ *(BIG),* ◊ *(MED1),* ○ *(MED2),* ▲ *(SMA).*

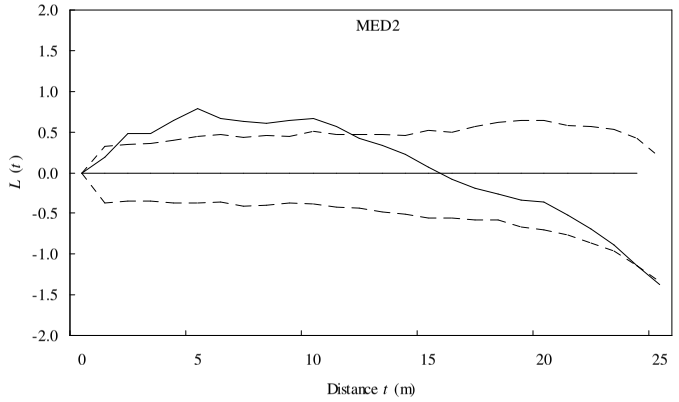
FIG. 3. *Variation of the L(t)-function according to distance (t) in four diameter classes of P. spruceanum. The lines correspond to the observed data. Broken lines correspond to the 95 % confidence intervals computed by Monte Carlo simulations under the hypothesis of complete spatial randomness.*

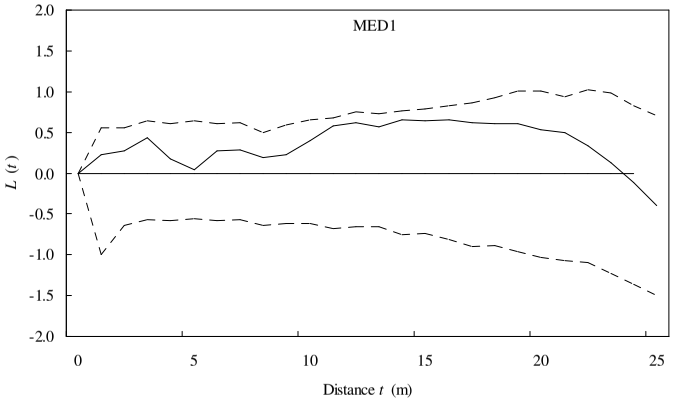
FIG. 4. *Spatial genetic structure according to distance in four diameter classes of P. spruceanum. Dotted lines represent upper and lower 95 % CIs around zero relatedness.*

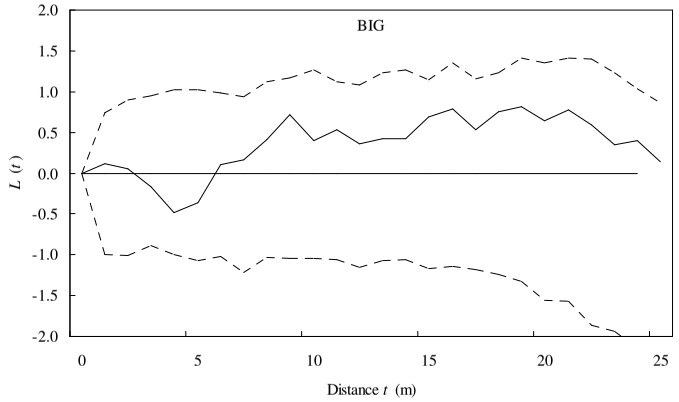

600
500
400
300
200
100
0
Number of individuals
SMA
MED2
MED1
BIG
0.06 to 3.02
(d.s.h.)
0.08 to 8
8 to 16
16 to 32
32 to 64
Diameter class (cm)

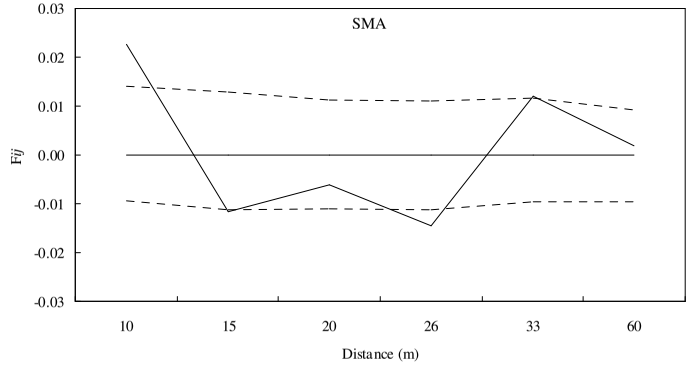

45
40
35
30
25
20
15
10
5
0
-5
(m)
0
5
10
15
20
25
30
35
40
45
50
55
60
65
(m)

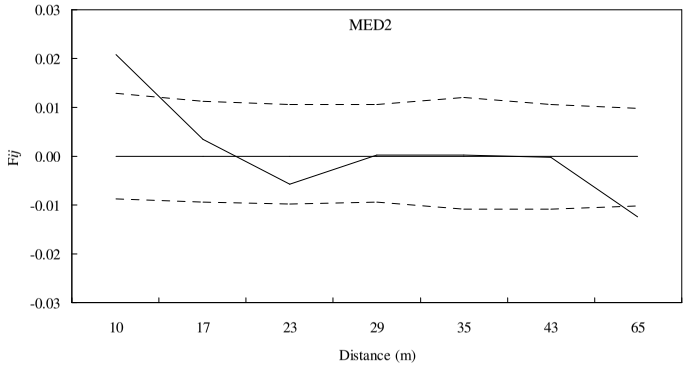

SMA
8.0
6.0
4.0
2.0
0.0
-2.0
-4.0
-6.0
-8.0
L (t)
0
5
10
15
20
25
Distance t (m)

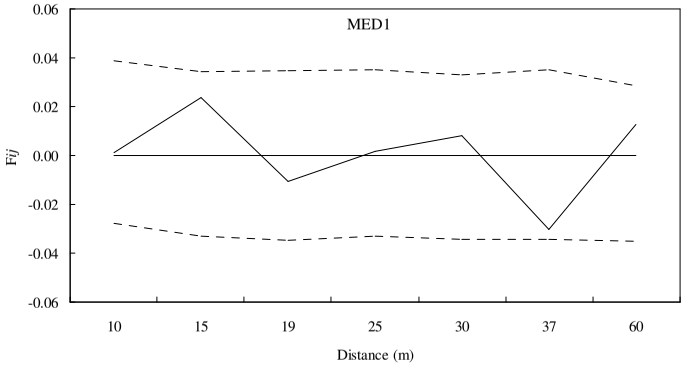

MED2
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
L (t)
0
5
10
15
20
25
Distance t (m)

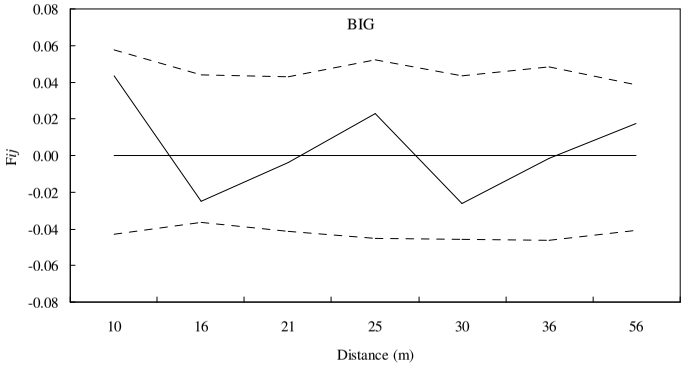

MED1
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
L (t)
0
5
10
15
20
25
Distance t (m)


BIG
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
L (t)
0
5
10
15
20
25
Distance t (m)


SMA
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
Fij
10
15
20
26
33
60
Distance (m)


MED2
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
Fij
10
17
23
29
35
43
65
Distance (m)


MED1
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
Fij
10
15
19
25
30
37
60
Distance (m)


BIG
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.08
Fij
10
16
21
25
30
36
56
Distance (m)

Supplementary Information. *Distribution of the all individuals of P. spruceanum found in the census within a 24 adjacent (100 m^2) sample plots. SMA, dsh 0.06–3.02 cm, N = 524; MED2, dbh 0.8–8 cm, N = 133; MED1, dbh 8–16 cm, N = 85; BIG, dbh > 16 cm, N = 50*

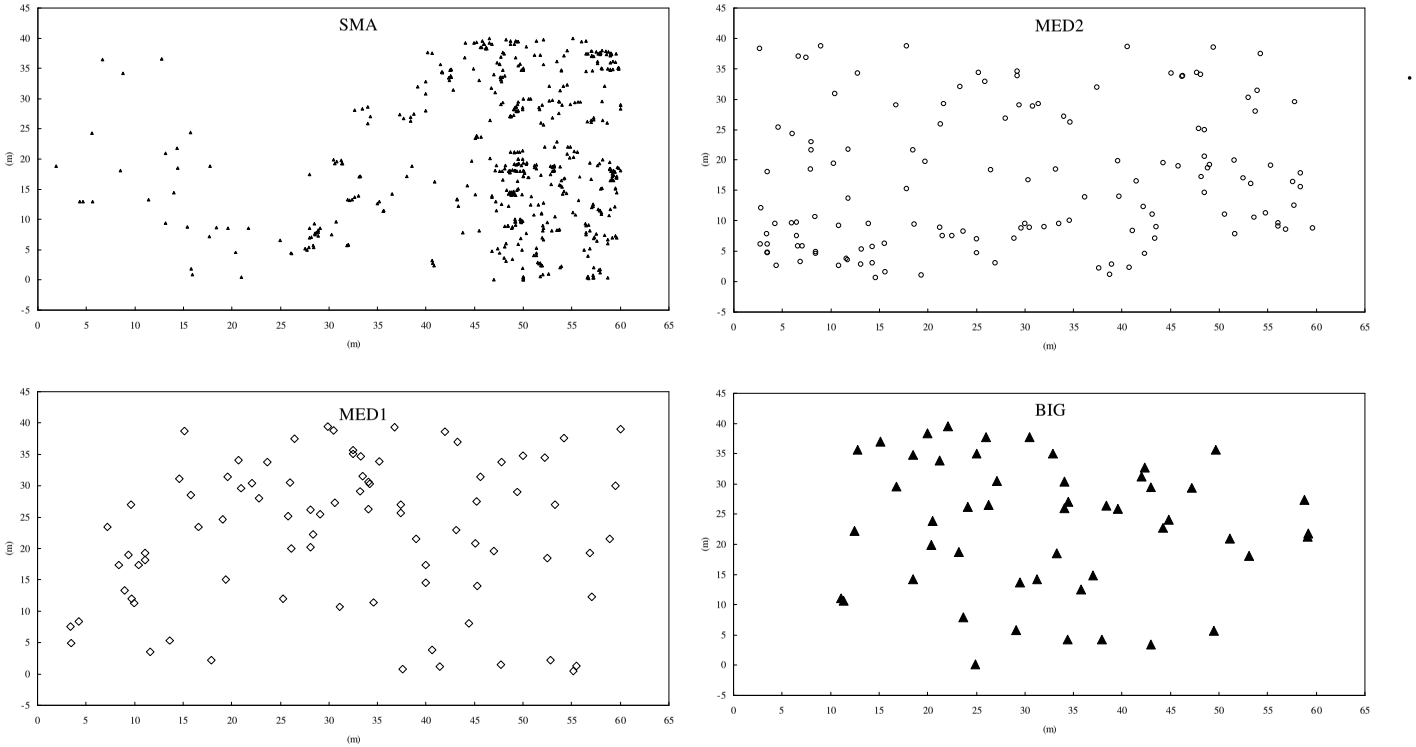


TABLE 1. *Population genetic statistics for four diameter classes from the population of P. spruceanum in the 2400 m^2 undisturbed primary forest sampled in Lavras, southeastern Brazil. Shown are the cohorts' codes, number of individuals' census, density of cohorts' census and number of genotyped individuals*

Cohort	N° ind.	Density (N ha^{-1})	Genotyped (% of census)	H_o (s.e.)	H_e (s.e.)	f (95% *C.I.*)
BIG	50	195.8	45 (90.0)	0.518 (0.012)	0.454 (0.004)	–0.143* (–0.185, –0.102)
MED1	85	354.2	60 (70.6)	0.467 (0.017)	0.434 (0.011)	–0.077* (–0.150, –0.023)
MED2	133	554.2	120 (90.2)	0.519 (0.017)	0.429 (0.013)	–0.211* (–0.287, –0.135)
SMA	524	2183.3	120 (22.9)	0.504 (0.026)	0.437 (0.007)	–0.153* (–0.240, –0.050)
Total	792	3287.5	345	0.504 (0.013)	0.438 (0.005)	–0.159* (–0.204, –0.097)

H_o = observed heterozygosity; H_e = gene diversity; f = inbreeding coefficient. * significant at the 5% level.

TABLE 2. *Differences in levels of gene diversity, inbreeding and genetic differentiation among cohorts as measured by H_e, f and F_{ST}, based on Goudet's G-test. Levels of significance were obtained after 6000 permutations using FSTAT (Goudet, 2002)*

Comparisons	Mean Diff. (H_e)	Mean Diff. (f)	F_{ST}
BIG vs. MED1	0.446 ns	–0.105 ns	0.004 ns
BIG vs. MED2	0.440 ns	–0.181 ns	0.003 ns
BIG vs. SMA	0.446 ns	–0.148 ns	0.004 ns
MED1 vs. MED2	0.430 ns	–0.147 ns	0.014 ns
MED1 vs. SMA	0.436 ns	–0.113 ns	0.001 ns
MED2 vs. SMA	0.430 ns	–0.190 ns	0.010 *

*$P < 0.01$; ns, not significant.

Supplementary Information. *Allelic frequencies for ten polymorphic loci for the four cohorts of P. spruceanum surveyed in this study.*

		Cohort			
Locus	Allele/*N*	SMA	MED2	MED1	BIG
Acp	1	0.705	0.523	0.773	0.648
	2	0.295	0.477	0.227	0.352
	N	105	107	55	44
Adh	1	0.709	0.679	0.664	0.670
	2	0.291	0.321	0.336	0.330
	N	115	112	58	44
Gdh	1	0.641	0.603	0.672	0.651
	2	0.359	0.397	0.328	0.349
	N	110	117	58	43
Gldh	1	0.706	0.685	0.664	0.674
	2	0.294	0.315	0.336	0.326
	N	114	111	58	43
Gtdh	1	0.676	0.704	0.686	0.670
	2	0.324	0.296	0.314	0.330
	N	111	115	59	44
Mdh 2	1	0.614	0.680	0.669	0.625
	2	0.386	0.320	0.331	0.375
	N	105	114	59	44
Per 1	1	0.675	0.769	0.737	0.689
	2	0.325	0.231	0.263	0.311
	N	106	119	59	45
Per 2	1	0.705	0.686	0.650	0.644
	2	0.295	0.314	0.350	0.356
	N	112	118	60	45
Sdh	1	0.654	0.731	0.695	0.659
	2	0.346	0.269	0.305	0.341
	N	114	119	59	44
Skdh	1	0.690	0.731	0.614	0.659
	2	0.310	0.269	0.386	0.341
	N	113	117	57	44

CONSIDERAÇÕES FINAIS

A noção de conectividade é de suma importância quando se considera que os corredores, como estruturas lineares de vegetação, são capazes de conectar os remanescentes isolados e diminuir os efeitos negativos da fragmentação, facilitando os fluxos de organismos, sementes e grãos de pólen. Entre as questões do presente estudo, procurou-se avaliar a estrutura genética de uma espécie arbórea com características ecológicas similares a de outros taxa comuns no sistema fragmentos-corredores de vegetação. Foram discutidas as implicações ecológicas dos resultados e elaborados argumentos alternativos para explicar a diversidade e diferenciação genética na referida paisagem. Em suma, nos corredores de vegetação secundária foram detectadas manutenção da diversidade genética, elevada identidade genética com os indivíduos dos fragmentos e ausência de endogamia. Portanto, nas condições em que o estudo foi realizado, os resultados sugerem a importância deste sistema na paisagem regional e a conservação dos corredores para a manutenção da diversidade genética. Os resultados são relevantes, pois os remanescentes de floresta estudados são 'hotspots' de biodiversidade (região de Floresta Atlântica do Brasil). Ressalta-se que, atualmente, estão sendo implementados corredores de conservação/fluxo gênico neste bioma, por agências de conservação nacionais e internacionais.

Outra questão investigada foi a estrutura genética no recrutamento das plantas, que poderia predizer a estrutura genética nas próximas gerações. Em escala espacial fina, a estrutura genética e demográfica evidenciou a relevância da conservação de pequenos fragmentos na paisagem, onde a diversidade genética pode ser mantida devido ao recrutamento natural pós-fragmentação, formando banco de plântulas com elevado conjunto gênico, importante aspecto para a conservação *in situ*. Aliado a isso, o estudo contribui para enriquecer o banco de dados sobre a estrutura genética espacial e demográfica de espécies arbóreas tropicais com alta densidade populacional polinizadas por insetos e dispersas por pássaros. Essas informações, sobretudo baseadas na estatística '*Sp*', podem ser úteis nas comparações quantitativas entre espécies com diferentes ou similares histórias de vida, quanto à coancestralidade espacial e entre diferentes estágios de vida. Em escala regional, tais informações serão uma referência útil na elaboração de estratégias de amostragem visando outros estudos em escala espacial fina ou, mesmo, na mensuração e na comparação dos níveis de diversidade genética em outras populações de *Protium spruceanum*.

Além disso, as informações obtidas no estudo da biologia reprodutiva serão relevantes para a continuidade dos estudos sobre fluxo gênico da espécie na paisagem, principalmente considerando que dados sobre o sistema de reprodução estão disponíveis apenas para algumas espécies de Burseraceae.

O aspecto funcional da conectividade no sistema fragmentos-corredores refere-se à resposta biológica peculiar de cada espécie à estrutura da paisagem e, por isso, novas hipóteses e a investigação em outras espécies com diferentes histórias de vida deverão fazer parte da continuidade dos estudos. Adicionalmente, estudos baseados em métodos diretos para estimar o fluxo gênico ao longo da paisagem são necessários para o entendimento da contribuição do fluxo de pólen e de sementes para a imigração contemporânea de alelos. Assim, os próximos estudos deverão, basicamente, responder às seguintes perguntas: Os corredores foram colonizados por fragmentos adjacentes? Quais são as estimativas de fluxo gênico contemporâneo ao longo do sistema fragmentos-corredores de vegetação? Como os corredores de vegetação afetam o comportamento dos pássaros? Como eles podem alcançar longas distâncias, a recolonização dos corredores é resultado de suas viagens entre os fragmentos? Como é a estrutura genética em espécies com características diferentes de história de vida? Qual é a estruturação genética em fragmentos que não são conectados por corredores de vegetação? Somente o acúmulo de informações sobre diferentes grupos de espécies poderá apontar direções mais sustentáveis para o manejo dos fragmentos, em que as escalas de espaço e de tempo são fatores relevantes que não devem ser desconsiderados.