MILENE SILVESTRINI

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UNIVERSIDADE ESTADUAL DE CAMPINAS
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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do Título de Doutor em Ecologia.

Supervisor/*Orientador*: FLAVIO ANTONIO MAËS DOS SANTOS
Co-supervisor/*Coorientadora*: MARIA IMACULADA ZUCCHI

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL
TESE DEFENDIDA PELA ALUNA
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ABSTRACT

The spatial genetic structure of plant populations may vary across life stages, across generations and among different environmental conditions. These changes are driven by evolutionary and ecological forces. Pioneer tree species exhibit particular life histories and population structures that are mainly affected by environmental changes generated by natural or human disturbances. Our aim was to investigate how the life-history traits, ecological processes, and the genetic factors associated to natural and human disturbances can affect the genetic diversity and structure of populations of a pioneer tree species. We studied *Croton floribundus* Spreng. (Euphorbiaceae), a pioneer tree species abundant in gaps and secondary areas of the semi-deciduous tropical forest, in two areas with contrasting levels of human disturbance: a primary forest and an early successional forest. In order to address the main question of this study, we examined the pattern of distribution of the species under the different environmental conditions generated by natural and human disturbances (Chapter I); tested and characterized universal chloroplast microsatellite (cpSSR) primers for *C. floribundus* (Chapter II); developed and characterized nuclear microsatellite (SSR) markers for *C. floribundus* as well as examined some cytogenetic traits of the species in order to test for polyploidy and to evaluate its implications for the appropriate use of the SSR markers (Chapter III); and evaluated the genetic diversity and structure of *C. floribundus* between two size classes and among populations in the primary forest and in the early successional forest (Chapter IV). *C. floribundus* was widespread and equally distributed along the gap size range in the primary forest, but its population structure varied between areas with contrasting levels of human disturbance. Six universal cpSSR loci were optimized and characterized for *C. floribundus*. The cytogenetic study allowed the accurate characterization of SSR loci as well as provided new data on the origin and evolution of the species. The number of bivalents observed in meiosis n=56 (2n=8x=112) showed the occurrence of polyploidy in all populations studied. High genetic diversity levels were found for *C. floribundus*. Seed dispersal and colonizations (and extinctions) were determinants of the fine-scale genetic structure of *C. floribundus* in both forest types. Also, their effects associated to the human disturbances seem to strongly increase the

genetic differentiation among populations in the early successional forest. Analysis of nuclear and chloroplast markers suggested that gene flow by pollen is responsible for maintaining the genetic diversity within populations of *C. floribundus* in both primary and early successional forests. In the latter, gene flow by seeds seem to be equally important. The results showed that gap dynamics, colonization process, and pollen and seed dispersal affect the genetic diversity and structure of the pioneer tree species by increasing or decreasing them depending mainly on the number of colonizers, the number of source populations, the gene flow rates, and the level of human disturbance of the area.

RESUMO

A estrutura genética espacial de populações de plantas pode variar ao longo dos estádios ontogenéticos, através das gerações e entre diferentes condições ambientais. Estas mudanças são direcionadas por fatores ecológicos e evolutivos. As espécies pioneiras apresentam histórias de vida e estruturas populacionais características que são afetadas principalmente pelas mudanças ambientais geradas por distúrbios naturais ou antrópicos. O objetivo deste trabalho foi investigar como as características do ciclo de vida, os processos ecológicos e fatores genéticos associados aos distúrbios afetam a diversidade e estrutura genética de populações de uma espécie arbórea pioneira. Nós estudamos *Croton floribundus* Spreng. (Euphorbiaceae), uma espécie arbórea pioneira abundante em clareiras e em áreas secundárias da Floresta Estacional Semidecidual, em duas áreas com níveis contrastantes de distúrbios antrópicos: uma floresta primária e uma floresta secundária em estádio inicial de sucessão. A fim de abordar a principal questão deste estudo, nós avaliamos o padrão de distribuição da espécie sob as diferentes condições ambientais geradas por distúrbios naturais e antrópicos (Capítulo I); testamos e caracterizamos iniciadores universais cloroplastidiais (cpSSR) para *C. floribundus* (Capítulo II); desenvolvemos e caracterizamos marcadores microssatélites nucleares (SSR) para *C. floribundus* bem como examinamos algumas características citogenéticas da espécie com o objetivo de testar a ocorrência de poliploidia e avaliar sua implicação para o uso dos marcadores SSR (Capítulo III); avaliamos a diversidade e estrutura genética de *C. floribundus* entre duas classes de tamanho e entre populações em uma floresta primária e uma floresta secundária em estádio inicial de sucessão (Capítulo IV). *C. floribundus* foi frequente e igualmente distribuído em clareiras de todos os tamanhos na floresta primária, mas sua estrutura populacional variou entre áreas com níveis contrastantes de distúrbio antrópico. Seis locos cpSSR foram otimizados e caracterizados em *C. floribundus*. O estudo citogenético permitiu a caracterização mais precisa dos locos SSR, bem como forneceu novos dados sobre a origem e a evolução da espécie. O número de bivalentes observados na meiose, n = 56 (2n = 8x = 112), mostrou a ocorrência de poliploidia em todas as populações estudadas. Altos níveis de diversidade genética foram encontrados para *C. floribundus*. A dispersão de sementes e as colonizações (e extinções) foram determinantes para a estrutura genética em fina escala encontrada nas populações de *C. floribundus* em ambos os tipos de florestas. Além disso, os efeitos

destes processos associados aos distúrbios antrópicos parecem aumentar fortemente a diferenciação genética entre as populações na floresta em estádio inicial de sucessão. As análises de marcadores moleculares nucleares e cloroplastidias sugeriram que o fluxo gênico por pólen é responsável por manter a diversidade genética dentro das populações de *C. floribundus* tanto na floresta primária quanto na floresta secundária em estádio inicial de sucessão. Nesta última, o fluxo gênico por sementes parece ser igualmente importante. Os resultados obtidos mostraram que a dinâmica de clareiras, o processo de colonização e a dispersão de pólen e sementes afetam a diversidade e estrutura genética da espécie arbórea pioneira, aumentando-os ou diminuindo-os conforme o número de colonizadores, número de populações-fonte, as taxas de fluxo gênico e o nível de perturbação antrópica da área.

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"Desvendando, paulatinamente, as sublimes grandiosidades da natureza invisível, a Ciência embriagou-se com a beleza de tão lindos mistérios e estabeleceu o caminho positivo de encontrar DEUS".

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INTRODUCTION

Genetic structure of populations refers to the distribution of genetic diversity within and among populations or the nonrandom distribution of alleles or genotypes in space and time (Loveless & Hamrick 1984). Because of the limited mobility of plants, genetic structure implies spatial structure. Changes in the spatial genetic structure of plant populations may occur across successive life stages, across generations and among different environmental conditions (Loveless & Hamrick 1984; Alvarez-Buylla et al. 1996; Jones & Hubbell 2006). These changes are driven by evolutionary and ecological forces. The evolutionary forces include genetic factors such as mutation, migration, drift and natural selection. The ecological forces include all processes that affect the demography of species, i.e., that involve or are associated with the survival and reproduction of species, determining their life histories (Loveless & Hamrick 1984; Ellstrand & Elam 1993; Alvarez-Buylla et al. 1996; Jones & Hubbell 2006). In tropical forest domains these processes are related mainly to patch dynamic regimes, biotic interactions and level of human disturbance. Understanding the effects of all these factors on the genetic diversity and structure of populations is important when defining strategies of forest conservation and restoration whose goal is to guarantee the maintenance of evolutionary and ecological dynamics as similar as possible to the natural conditions (Loveless & Hamrick 1984; Ellstrand & Elam 1993).

Pioneer tropical tree species exhibit particular life histories and population structures that are mainly affected by the patchy dynamics of the tropical forests (Swaine & Whitmore 1988; Alvarez-Buylla 1994; Uriarte et al. 2012). Seeds of pioneer species can “germinate only in those canopy gaps open to the sky in which

full sunlight impinges at ground level for at least part of the day" (Swaine & Whitmore 1988; Whitmore 1989). Pioneer species usually have seed banks and show long distance seed dispersal (Whitmore 1983; Swaine & Whitmore 1988, Uriarte et al. 2012). In primary or late successional forests, demographic dynamics of the species may resemble those defined for a metapopulation, i.e., the populations persist at a regional scale as a result of a balance between the processes of local population extinction, patch migration, and colonization (Levins 1969; Hanski & Gilpin 1991; Freckleton & Watkinson 2002). All these characteristics have significant consequences for genetic structure and evolution, however, this is still poorly understood.

According to Wright (1940) and Loveless & Hamrick (1984) we might expect a clear population structuring in pioneer species because the number of gap colonizers is generally small (a founder effect) and due to the fixation of neutral alleles by genetic drift. Conversely, seed banks and long distance seed and pollen dispersal could homogenize spatial genetic variation (Loveless & Hamrick 1984; Alvarez-Buylla & Garay 1994). Besides, under some conditions, the extinction and recolonization processes can act as a form of gene flow reducing genetic divergence among populations (Slatkin 1977, 1985; Wade & McCauley 1988; Whitlock & McCauley 1990). In a metapopulation context, processes associated with extinctions and recolonizations as mentioned above and others such as origin of gap founders, population size and age may also affect their genetic diversity and structure (Whitlock & McCauley 1990; McCauley et al. 1995).

Studies that have examined the genetic diversity and structure of pioneer species have shown two opposite sets of results: nonstructured (Alvarez-Buylla et

al. 1996; Ribas & Kageyama 2004a; Davies et al. 2010) and genetically structured populations of adults (Kageyama et al. 2003; Ribas & Kageyama 2004b; Jones & Hubbell 2006; Born et al. 2008a). However, the same studies agreed with the high level of genetic diversity of the populations and the loss of genetic structure across successive life stages. It is important to note that only a few species in a range of gap-phase regeneration behavior of the pioneer species were evaluated (Brokaw 1987), mainly those restricted to large gaps and that colonize only when the gap is still very open (for example, see Alvarez-Buylla et al. 1996; Ribas & Kageyama 2004a,b).

The results of Alvarez-Buylla et al. (1996) supported the hypothesis that patchy recruitment in gaps, or among gap heterogeneity, influences the genetic structure of the pioneer *Cecropia obtusifolia* Bertol. (Cecropiaceae) in a pristine forest. On the other hand, the strong spatial genetic structure seen in seed rain and seedlings decreased in successive life stages and was not found in adults and soil seeds. Ribas & Kageyama (2004a) also found loss of genetic structure from the seedling stage to adults of the pioneer *Trema micrantha* (L.) Blume (Ulmaceae) and Jones & Hubbell (2006) found the same from seed to sub-adult individuals of *Jacaranda copaia* (Aubl.) D. Don (Bignoniaceae). Alvarez-Buylla et al. (1996) suggested that the loss of genetic structure across successive life stages was due to the random mortality during gap thinning or to the occurrence of selection favoring heterozygote survival in gaps.

In early successional forests, these species are expected to exhibit a different spatial genetic structure since their patterns of distribution, survival and reproduction change due to the site's microclimate and biota modifications after the

human disturbance (Matthes 1992; Danciguer 1996; Martínez-Garza & Howe 2003; Swanson et al. 2011; Tabarelli et al. 2012). Depending on the level and type of human disturbance in the site, the environmental heterogeneity and differential selection pressures created might result in demographical and genetic changes. Also, differently from primary or late successional forests, the populations in the early successional forests have still not undergone extinctions, but are influenced primarily by colonization processes (Swanson et al. 2011). Thus, factors related to the establishment of the species in the site, such as origin and number of propagules as well as the level of gene flow, either from primary or secondary forests, may determine the demography and the genetic diversity and structure of the species.

Availability of local plant propagules to the species reestablishment in disturbed sites depends on the severity of the disturbance (Uhl et al. 1982; Uhl & Jordan 1984; Uhl et al. 1991). Studies of plant succession in Amazonia (Uhl et al. 1982, Uhl & Jordan 1984, Uhl et al. 1991) pointed out that in sites with a light land use, e.g., following forest cutting and burning and the abandonment after a short period of grazing, pioneer species were recruited from sprouts, seed banks or dispersed seeds. In more severe disturbances as forest cutting and burning followed by several years of farming, sprouts were rare because frequent farm plot weeding exhausted sprouting reserves and the tree establishment depended on neighboring seed sources. Therefore, in this case, the genetic diversity and structure of recent established populations would be more affected by colonization and the level of gene flow with other populations since the local populations were extinct.

Uhl et al. (1991) showed that after intensive land use, the community of herbivores and predators of seeds and seedlings is very different from primary forests or less intensive land use sites, affecting the establishment of forest species. Another important factor that may influence the genetic parameters of pioneer trees in these sites is the strong competition with grasses and alien shrubs (Uhl et al. 1991; Holl et al. 2000).

There are few studies that consider the genetic diversity and structure of pioneer species in early successional forests. The results have suggested that gene flow and origin of populations are the determinant factors of the genetic patterns of diversity and structure that were found (Alvarez-Buylla & Garay 1994; Franceschinelli & Kesseli 1999; Cavers et al. 2005; Born et al. 2008b; Davies *et al.*, 2010; 2013). It seems that the founder effects (Cavers et al. 2005; Franceschinelli & Kesseli 1999) and the aggregate distribution of individuals due to a limited seed dispersal (Born et al. 2008b) could increase endogamy and generate genetically structured populations in early successional forests. However, some of these studies have examined genetic differentiation among populations of forests with different levels of human disturbance and/or succession stages combined as a unique pool of populations. For this reason, more specific information about genetic diversity and fine-scale structure of populations of pioneer species in early successional forests is required. Furthermore, it is important to distinguish the effects of colonization and level of human disturbance on the genetic parameters.

Croton floribundus Spreng. (Euphorbiaceae) is a shade-intolerant, fast-growing pioneer tree species that occurs abundantly in gaps of primary remnants as well as in secondary areas of the semi-deciduous tropical forest (IBGE 1992) of

the countryside of São Paulo State, as well as other forests of Brazil and eastern Paraguay (Lorenzi 1992; Gandolfi et al. 1995; Rodrigues 1999; Rodrigues 2005; Gomes 2006). The high abundance of plants in gaps are an important characteristic of the spatial structure of *C. floribundus* because they help to find the minimum sampling number required for appropriate analysis of the genetic structure among populations in gaps, a fundamental question in population genetics of pioneer species. Moreover, the reproductive system of *C. floribundus* is relatively well known, which facilitates the association of genetic and demographic processes. The trees are monoecious self-compatible (Passos 1995). Dichogamy is much accentuated and seems to reduce self-fertilization (Passos 1995). The species also presents no seed bank (Lorenzi 1992; Grombone-Guaratini 1999; Carvalho 2001) and no asexual reproduction (Passos 1995).

C. floribundus has explosive seed dispersal (autochory) (Lorenzi 1992) and is pollinated by flies and wind (Passos 1995). Pioneer insect-pollinated trees have shown high gene flow rates; however, most of them also have long-distance seed dispersal by wind or birds (Litrico et al. 2005; Born et al. 2008a). Thus, a fine-scale study of genetic diversity and structure of this species using nuclear and chloroplast markers may help to elucidate how these mechanisms of dispersal with opposite effects on gene flow rates affect the distribution of the genetic variation in the species.

We believe that a comparison between populations of *C. floribundus* located in primary and early successional forests where colonization and extinction processes, as well as level of human disturbance, differ could shed light on what are the determinant factors of the patterns of genetic diversity and structure in this

species. Additionally, a detailed analysis of the genetic diversity parameters among life stages or size classes can provide key information needed to understand the genetic dynamics of the populations. Because of the features presented above and the others analyzed in this study, we believe *C. floribundus* may be an excellent model for the pioneer species with similar modes of colonization and life histories. The knowledge of such aspects of the pioneer species is very important for understanding the human disturbance effects and the successional processes in the tropical forests, which in turn is important to the forest conservation, management and restoration.

The main aim of this study was to investigate how the ecological processes and the population genetic factors associated with disturbances can affect the genetic diversity and structure of populations of the pioneer tropical tree species *C. floribundus*. In order to achieve this aim, other specific objectives were set as follows:

1) to evaluate the distribution of individuals of *C. floribundus* in the gaps of a primary forest and compare the plant density of two size classes in a primary and in an early successional forest in order to test for differential plant distribution under the variable environmental conditions generated by natural or human disturbances (Chapter I);
2) to test and characterize chloroplast microsatellite primers for *C. floribundus* (Chapter II; Cordasso 2011);
3) to develop and characterize nuclear SSR markers for *C. floribundus* and to examine the cytogenetic traits of the species in order to test for the

occurrence of polyploidy in the species and to evaluate its implications for the appropriate use of the SSR markers (Chapter III);

4) to evaluate the genetic diversity and structure of the pioneer tree *C. floribundus* between two size classes and among populations in a primary forest and an early successional forest. Specifically, we attempted to answer the following questions: 1) Do the genetic diversity and fine-scale genetic structure of *C. floribundus* differ between primary and early successional forests? 2) Are there changes in the genetic diversity and structure between size classes in these forests? 3) Is there a correspondence between the genetic structure of populations in the primary forest and their spatial distribution in the gaps? 4) How do ecological processes and genetic factors associated with natural and human disturbances affect the genetic diversity within and among populations in the species? (Chapte IV).

CHAPTER I

Variation in the population structure of a pioneer tree species under natural and human disturbances

Variation in the population structure of a pioneer tropical tree species under natural and human disturbances

Article according to instructions of Ecology and Evolution (under revision)

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Abstract

The distribution of tree species in tropical forests is generally related to the occurrence of disturbances and local availability of resources, primarily light. Thus, the distribution of pioneer tree species is expected to vary according to the gap characteristics and level of human disturbance. We asked whether there was variation in the distribution of a pioneer species under different environmental conditions generated by natural and human disturbances. To answer this question, we studied the distribution patterns and population persistence of the pioneer tree species *Croton floribundus* in the size and age gap range of a primary Brazilian forest. Additionally, we compared the plant density of two size classes between a primary and an early successional human disturbed forest. *Croton floribundus* was widespread and equally distributed along the gap-size gradient in the primary forest. Overall density did not vary with gap size or age (F-ratio=0.062, p=0.941), and while juveniles were found to have a higher density in the early successional forest (p=0.021), tree density was found to be similar between forests (p=0.058). Our results indicate that the population structure of a pioneer tree species with long lifespan and a broad gap-size niche preference varies according to the level of human disturbance, but not with the level of natural disturbance. We believe this can be explained by the extreme environmental changes that occur after human disturbance. The ecological processes that affect the distribution of pioneer species in natural and human modified forests may be similar, but these results suggest they act differently under the contrasting environmental conditions generated by natural and human disturbances.

Key words: environmental heterogeneity, canopy gaps, population structure, semi-deciduous tropical forests, succession

Introduction

Pioneer species are thought to present different population structures under different stages of forest succession or levels of human disturbances (Matthes 1992; Swanson et al. 2011; Tabarelli et al. 2012). In primary or late successional forests, population dynamics of the species may resemble those defined for a metapopulation, i.e., the populations persist at a regional scale as a result of a balance between the processes of local population extinction, patch migration, and colonization (Levins 1969; Hanski and Gilpin 1991; Freckleton and Watkinson 2002). In early successional forests grown after human disturbances, pioneer patterns of distribution, survival and reproduction must change due to the shifts in the local environmental conditions such as light, temperature, as well as biotic factors (Matthes 1992; Martínez-Garza and Howe 2003; Swanson et al. 2011, Tabarelli et al. 2012, Lohbeck et al. 2014). These expectations are based on the exploiter-mediated coexistence model in patches, which states that coexistence of tree species in tropical forests, and therefore the distribution of their populations, relies on the differential availability of resources and space generated by gap disturbance regime (Connell 1978; Paine and Levin 1981; Begon et al. 2006; but see Hubbell et al. 1999). Thus, in a primary forest, the metapopulation structure of pioneer trees would be the result of the patchy recruitment generated by treefall gap disturbances and, in an early successional human disturbed forest, tree species would also respond to variation in resources availability by changing their demographic characteristics according to the new environmental conditions of the area.

Recent studies have found an incredibly high enhancement in plant density of pioneer species under human modified environments such as the edges of tropical

fragmented forests when compared to the forest interior (Laurance et al. 2006; Santos et al. 2008; Santos et al. 2012). The forest edges would work as the early successional forests due to the similarity of environmental conditions in the areas, mainly elevated light availability (Tabarelli et al. 2008; Santos et al. 2012). Despite the consensus in the response among pioneer species regarding the juvenile abundance, long-lived pioneer species exhibited populations with negative adult recruitment along the forest edges (Santos et al. 2012). According to Santos et al. (2012), the reason was probably the adult sensitivity to edge effects such as wind and physiological stress. However, the existence of an underlying pattern for the distribution of long-lived and short-lived pioneer species under these conditions remains unclear. Furthermore, there are still few empirical data available from early successional forests.

In well-conserved or late successional forests, pioneer density can vary greatly among gaps depending on the species (Brokaw 1987). It seems that some pioneer species have a narrower niche preference than others, such as *Trema micrantha* (L.) Blume and *Trema orientalis* (L.) Blume (Ulmaceae). These species occupy preferably, have high plant density, and reproduce only in large gaps (above $400m^2$) or in high light environments (Brokaw 1987; Goodale et al. 2012). The higher plant density in large gaps and in edge-affected habitats and/or early successional forests would be a result of the fine specialization to the high availability of resources, mainly light, in these areas by these short-lived, rapid-colonizers pioneer species (Brokaw 1987; Goodale et al. 2012). Such high level of resources must sustain the higher and faster growth of these species (Bazzaz and Pickett 1980; Brokaw 1987). In contrast, some pioneer species seem to be able to survive and reproduce across wider ranges of gap size and light conditions (Brokaw 1987; Goodale et al. 2012). This is the case of *Miconia argentea* (Sw.) DC. (Melastomataceae), a

relatively "shade tolerant" pioneer species that is found in a broad range of gap sizes (from 100 m^2 to 705 m^2), and therefore, has a large gap-size niche preference (Brokaw 1987).

Croton floribundus Spreng. (Euphorbiaceae) is a shade-intolerant, fast-growing pioneer tree species (Lorenzi 1992; Gandolfi et al. 1995; Rodrigues 1995) that presents high photosynthetic rates (M. Silvestrini and I.F.M. Válio, unpublished data; Ribeiro et al. 2005) and germinates only under alternating temperatures (Válio and Scarpa 2001). It is abundant in gaps of primary remnants as well as in secondary areas of the semi-deciduous tropical forest (Lorenzi 1992; Gandolfi et al. 1995; Rodrigues 2005). These features seem to be directly related to a narrow niche preference: large gaps or high light environments. Thus, one can expect higher plant densities of both reproductives and juveniles of this species in early successional habitats rather than in natural or primary forests. Likewise, both size classes must be found mostly in the large gaps of the primary forest, because only in these sites there would be the level of resources required by the rapid growth of the species. However, the species presents some unusual pioneer tree characteristics such as relatively large seeds (Válio and Scarpa 2001), autochorous seed dispersal syndrome (Lorenzi 1992), no seed banks (Lorenzi 1992; Carvalho 2001) and no asexual reproduction (Passos 1995; Danciguer 1996), which may demonstrate a different ecological behavior from the traditionally studied pioneer species restricted to large gaps. In addition, there are known to be higher height and larger diameter plants of this species in a primary forest (Rodrigues 2005; T.E. Barreto, unpublished data) than in secondary forests (Lorenzi 1992; Danciguer 1996), which may suggest that the species is long-lived and not short-lived as it has been generally considered (Lorenzi 1992; Danciguer 1996).

Here, we proposed to evaluate the distribution of individuals of *C. floribundus* in the gaps of a primary forest and compare the plant density of two size classes in a primary and

in an early successional forest in order to test for differential plant distribution under the variable environmental conditions generated by natural or human disturbances. Additionally, we examined the population persistence of the species and the relation between size class and reproductive activity in the primary forest, where larger sized plants were observed compared to secondary forests. The following questions were addressed: (i) Does *C. floribundus* have a large, intermediate or narrow gap-size niche preference in the primary forest? (ii) Does the pioneer species present the same plant density between the natural and human modified environments of a primary and an early successional forest? (iii) Is there difference in occupation of the space by juveniles and trees of the pioneer species between forest types? (iv) Is *C. floribundus* a long-lived pioneer species?

Materials and methods

Study site

The study was conducted in a 10.24 ha permanent plot in the primary forest at Caetetus Ecological Station (CES) (Rodrigues 2005), and in an early successional forest adjacent to the state reserve at Torrão de Ouro Farm (Fig. 1). CES is located in Gália and Alvinlândia, state of São Paulo, southeast Brazil (22°20' - 22°30'S; 49°40' - 49°45'W). Altitude ranges from 520 to 680 m (Tabanez et al. 2005). The climate at CES region corresponds to Köppen's "Cwa" mesothermic type, that is, humid subtropical with a dry winter (Rodrigues 2005; Tabanez et al. 2005). Annual rainfall averages 1,431 mm yr^{-1} and average annual temperature is 21.5 ºC.

CES consists of 2,178.84 ha of semi-deciduous tropical forest or the premontane moist forest according to Holdridge (1967). This forest originally covered almost all the plateau in the state of São Paulo. Nowadays, it is the most threatened forest of São Paulo

due to the historic fragmentation and deforestation that occurred since the beginning of European colonization in Brazil (16th century). Most of the area of CES is covered by well conserved vegetation. The forest at CES is very dynamic, with larger gaps, and gap density and total percent of gap area higher than other tropical forests (Lima et al. 2008; Martini et al. 2008). According to historical reports dating from the early 20th century, most of the area including the center of the reserve, where the 10.24 ha permanent plot was delimited, has not experienced anthropogenic disturbances. On the other hand, the edges of the reserve are very disturbed (Tabanez et al. 2005). The original forest in the selected site at Torrão de Ouro Farm was cleared in 1926 and converted to a pasture. In 1984, the site was abandoned and farmers stopped slashing, but cattle grazing was still allowed. In 1986, the vegetation was burned, and in 2008, the pasture was completely closed to cattle grazing, allowing forest recovery. By the time this study was conducted (2010), the site was covered by an early successional forest. Early successional forest is defined here as being a site completely deforested in the past followed by human land use along a period of time, abandonment after the end of human activities, and thence under natural regeneration process with typical dominance by few fast growing pioneer species. This forest differs from a primary forest mainly due to: i) lower number of species (dominance of pioneer tree species); ii) lower canopy height; and iii) openness and discontinuity of the canopy, which contribute to the formation of a brighter understory.

Soils in the early successional forest at Torrão de Ouro Farm are of the same type as at the CES plot, i.e., the red-yellow Acrisols (Ultisols) and Gleysols (Entisols) (Rodrigues 2005; M. Cooper, personal communication).

Study species

Croton floribundus Spreng. (Euphorbiaceae) is recognized as a pioneer species by Rodrigues (1995) and Gandolfi et al. (1995). It is a polyploid (Silvestrini et al. 2013), shade-intolerant, fast-growing tree species commonly found in gaps of primary remnants as well as in secondary areas of the semi-deciduous tropical forest (Lorenzi 1992; Gandolfi et al. 1995; Rodrigues 2005). The species range includes other forests in Brazil and in Eastern Paraguay (Lorenzi 1992; Gandolfi et al. 1995; Rodrigues 2005; Gomes 2006). According to Lorenzi (1992) and Danciguer (1996), tree height ranges from 4 to 13 m, but in the primary forest at CES, tree height ranges from 3 to 30 m (Rodrigues 2005; T.E. Barreto, unpublished data).

The seeds (5 x 4.5 mm) are much larger than other pioneer species and require alternating temperatures for germination in light as well as in darkness (Gomes 2006; Válio and Scarpa 2001). Seed dispersal occurs by autochory or ballochory, i.e., plants present explosive seed dispersal (Lorenzi 1992). *C. floribundus* has been found in seed banks in wet (Grombone-Guaratini 1999) and dry seasons (Carvalho 2001). However, the seeds are heavily attacked by predators (Carvalho 2001) and present a short period of viability, 3-4 months at most (Lorenzi 1992; M.T. Grombone-Guaratini, personal communication). Therefore, we assume that this species ability to persist long term in the soil seed bank to be very limited.

For this study the following size classes based on Danciguer's (1996) definition of life stages for *C. floribundus* were used: 1) seedlings (presence of cotiledonary leaves); 2) juveniles 1, hereafter referred to as juveniles, which consisted of individuals of 5 to 37 cm height and 0.05 to 0.40 cm diameter at soil height (DSH); 3) juveniles 2, which consisted of individuals of 27 to 400 cm height and 0.45 to 4.50 cm diameter at soil height (DSH); 4) trees, which comprised individuals > 4.78 diameter at breast height (DBH). The definition

of the tree size class was based on the criterion of inclusion of individuals in the plant inventory in the permanent plot (DBH ≥ 4.78 cm) (Rodrigues, 2005). Plant material of two reproductive trees from CES and one from Torrão de Ouro Farm is deposited at UEC Herbarium, University of Campinas, Campinas, Brazil (accession numbers 172052, 172054, and 172055, respectively).

Data analysis

Distribution of individuals in the gap range

Analysis of distribution of individuals of *C. floribundus* in the gaps of the primary forest were based on the previous characterization of the gaps in the permanent plot carried out by Lima et al. (2008), Martini et al. (2008), and A.M.Z Martini and R.A.F. Lima (unpublished data) (Fig. 2). Gap area data were based on the method of Brokaw (1982): "the vertical projection of the hole in the forest extending through all levels down to an average height of two meters above ground". Gap age classes were defined by Martini et al. (2008) as follows: Age 3 (old) = tree disturbance occurred before 2002; Age 2 (intermediate) = tree disturbance occurred after 2002; Age 1 (fresh) = tree disturbance occurred after 2002, but it is more recent than age 2 due to the presence of "bark and thin twigs on the terminal branches of the tree or the part of the tree (stem or branch)" (for further details, see Martini et al. 2008). We sampled sixteen gaps with different areas, ages and modes of disturbance, in which density and frequency of *C. floribundus* (individuals of all size classes) were evaluated. The gradient in gap size was from 23 m^2 to 645 m^2 and the total gap area sampled was 3,578 m^2.

Since the distribution of size class frequencies of canopy gaps followed the same pattern found by Lima et al. (2008) (data not shown), we considered our sampling

representative of the plot canopy gaps. Seven complex gaps, which mean gaps formed by distinct episodes of tree mortality, were evaluated. In these cases, disturbance age was based on the most recent episode.

A multiple regression was applied in order to test if gap area (X_1) and gap age class (X_2) contribute to explain plant density (Y) in gaps (Zar 2010). Firstly, the analysis was applied for individuals of all size-classes. Secondly, it was applied for each size-class (juveniles and trees) separately. Statistical differences among plant density in the gap age classes were checked by comparison of medians through the notched box plots (McGill et al. 1978).

Population persistence

Population persistence of *C. floribundus* was inferred based on the size distribution of trees censused in the plant inventories of the permanent plot in 2005 (Rodrigues 2005) and 2010 (T.E. Barreto, unpublished data). Size distribution of individuals was visualized through box plots.

Reproductive activity and tree size

In order to evaluate the relation between size and reproductive activity of the trees of *C. floribundus* in the permanent plot of the primary forest, we observed the presence of flowers in 131 trees at the beginning of December 2010, during the blooming period of the species (Lorenzi 1992; Passos 1995).

Simple logistic regression (Zar 2010) was applied to calculate the probability of encountering a tree in bloom at a given DBH in the population.

Plant density

Juvenile and tree densities (individuals m^{-2}) of *C. floribundus* in the early successional forest at Torrão de Ouro Farm were assessed in order to compare with the primary forest at CES. All juveniles and trees found in eight transects of 2 x 50 m systematically distant 40 m each other and distributed all over the forest area were censused (a total area of 800 m^2). In the CES permanent plot, juvenile density in the gaps was calculated based on the evaluation of *C. floribundus* distribution in the sixteen gaps from the gap range analysis (a total Brokaw gap area of 3,578 m^2). For trees, density was obtained dividing the number of individuals surveyed in each permanent subplot (T.E. Barreto, unpublished data) by the subplot area (400 m^2) (n=256) (a total plot area of 102,400 m^2). Since the sampled populations were very asymmetrical and sample sizes were different, mainly for trees, we did not perform tests for assumptions of the parametric statistic tests as recommended by Zar (2010). Yet, the variability for each of the samples was reported. Both an appropriate parametrical and a nonparametrical test, a two-sample t-test with separate variances and the Mann-Whitney test, respectively, were performed.

The simple logistic regression was performed using software R version 2.15 (R Development Core Team, 2010). All other statistical analyses were performed using Systat 11 software (Systat Software Inc., Richmond, CA).

Results

Distribution of individuals in the gap range

A total of 317 individuals (all size classes) of *C. floribundus* were found in the sampled gaps of the primary forest. Frequency of *C. floribundus* in the gaps was 94%. The species was found in gaps of all sizes (Table 1, Fig. 3a). We did not find the species only in

the gap H9 (Table 1, Fig. 2). This gap has disturbance modes, gap area, and gap age (Table 1) similar to the other gaps analyzed but, distinctively, it was not surrounded by any tree of *C. floribundus* in a minimum radius of 34 m (Fig. 2). Density of *C. floribundus* (all size classes) in the gaps was independent of gap size and gap age (F-ratio=0.062, p=0.941) (Fig. 3).However, the density of trees (DBH $\geq$ 4.78 cm) was higher in older gaps (age 3) (p=0.02, two-tail, gap age coefficient) (Fig. 4b), while juvenile density did not respond to neither gap area nor gap age class (F-ratio=0.435, p=0.657). Note that juveniles of *C. floribundus* were recorded even in old gaps, i.e., more than eight-year-old gaps (Table 1). On the other hand, there were no seedlings and practically no juveniles ($\leq$ 1 individual) in the same old gaps that contained trees, except for gaps where there were recurrent tree disturbances (Table 1). Also, the highest DBH of a tree recorded in a gap was of 20.85 cm.

Population persistence

The size distribution of individuals in the permanent plot of the primary forest at Caetetus Ecological Station (CES) showed that most of individuals are between 7.9 and 20.0 cm DBH (Fig. 5). However, 25% of individuals or more were larger than 20.0 cm DBH. Besides the high abundance in the populations, trees larger than 20.0 cm DBH had a lower growth rate than average size individuals (data not shown). This indicates that populations of *C. floribundus* persist for relatively long time after gap disturbances in the primary forest and the species has a relatively long life span.

Reproductive activity and tree size

Flowering trees were found in all evaluated sizes (from trees with 4.78 cm to 47.11 cm DBH) (Fig. 6). The estimated linear logistic model was the following:

$$ln\left(\frac{p}{1-p}\right) = -2.852 + 0.363 * DBH$$

where p is the probability of encountering a plant in bloom at a given size. Both logit coefficients were significant with $p<<0.0001$. The chances of reproduction were 90% for trees with DBH ≥ 13.9 cm and 99% for trees with DBH ≥ 20.5 cm (Fig. 6).

Plant density

Density of trees did not differ (p=0.058) between the early successional forest at Torrão de Ouro Farm (0.023 ± 0.008 ind m^{-2}, n=8) and the primary forest at CES (0.0050 ± 0.0005 ind m^{-2}, n=256). The Mann-Whitney test also showed statistical similarity of tree density between both forest types (p=0.058). Juveniles presented higher densities (p=0.021) in the early successional forest at Torrão de Ouro Farm (0.21 ± 0.06 ind m^{-2}, n=8) than in the gaps of the CES primary forest (0.04 ± 0.01 ind m^{-2}, n=16). A similar statistical result was found by the Mann-Whitney test (p=0.003).

Discussion

In contrast to the expectation based on the ecophysiological characteristics of the species, we found *C. floribundus* to be widespread and equally distributed along the gap-size gradient in the primary forest. Also, population persistence inferred by the size distribution of trees showed that the species might be long-lived under natural conditions and reach reproductive stage at small size classes (Poorter et al. 2006; Bentos et al. 2008). The species showed a regeneration behavior similar to the pioneer species *Miconia argentea* (Sw.) DC. (Melastomataceae) and opposite to *T. micrantha* and *T. orientalis* (Brokaw 1987; Goodale et al. 2012), species considered as the "endpoints on a scale of

regeneration behavior" (Brokaw 1987). Other demographic characteristics of *C. floribundus* that seem to be similar to *M. argentea* are plant recruitment and survival along the time after the gap creation. It was common to find seedlings, juveniles 1 and juveniles 2 coexisting in gaps but not trees with seedlings and juveniles. It is worth noting that there was a large variation in size of trees in old gaps, which is likely a result of this colonization in extended time. For instance, we found plants ranging from 4.78 cm to 15.60 cm DBH in a gap and 9.07 to 20.85 cm DBH in another.

During the gap analysis we verified that *C. floribundus*, mainly seedlings and juveniles, was also present under the canopy trees surrounding the openings. This along with the results discussed above may demonstrate a relatively higher shade-tolerance *C. floribundus* than other pioneer species as well as confirm its larger regeneration niche. Thus, the species seems to occupy a more heterogeneous environment. Such characteristics confirm that, as other regeneration classes of trees, pioneer species can also show a continuum of responses to irradiance (Brokaw 1987).

Despite the high forest turnover rate for the semi-deciduous tropical forest at CES (Lima et al. 2008) - 98 years considering Brokaw area and 38 years considering Runkle (1982) area - and the high frequency of recurrent disturbances (Lima et al. 2008), population persistence of *C. floribundus* in the gaps is high due to the survival of a few old remnant individuals in each gap. It seems that some individuals have a long reproductive phase, since reproduction may start at early life-stages or small size classes. On the other hand, earlier reproduction may be a selective advantage for a light-demanding pioneer species that also grows in small or medium gaps where rapid canopy closure can decrease chances of plant survival in late life-stages. Furthermore, a longer period of seed production can result in a higher number of seeds per individual. This could overcome the short-

distance seed dispersal by helping the species reach gaps in time instead of space. Such a strategy would be consistent with a large gap-size niche preference and a high forest turnover rate because any new opening gap would be a favorable regeneration site for the pioneer species.

Density of *C. floribundus* (all size classes) in the gaps of the permanent plot was unpredictable in function of gap size and age, but gap age affected the abundance of trees. This means that we cannot expect higher plant densities in the early successional forest as in a large gap or a high light environment (Brokaw 1987; Goodale et al. 2012; Santos et al. 2012). Indeed, even though the early successional forest presents the favorable light and temperature microclimate conditions for pioneer recruitment all over the area, especially in the beginning of colonization, tree density was similar between forest types. The reason can be the low number of colonizers in the area (Martínez-Garza and Howe 2003). The large vacant site of the early successional forest seems not to be easily reached by a short-distance dispersed tree as *C. floribundus* (Lorenzi 1992; Stamp and Lucas 1983; Passos and Ferreira 1996). Also, there might be barriers to plant recruitment and establishment owing to the land use and the modified ecological conditions in the area that decreased plant density such as cattle grazing (see land use history) and competition with grasses and weeds (Uhl et al. 1991; Holl et al. 2000). The long-term persistence of *C. floribundus* in the primary forest and the age of the early successional forest can account for the tree abundances in both forests, explaining the result as well. Our results confirmed previous studies, which have shown that for some pioneer species, specifically long-lived ones, only saplings and juveniles had increment in plant density at early successional or secondary environments (Danciguer 1996; Santos et al. 2012). Most importantly, the results showed that even for a pioneer species not restricted to large gaps, i.e., whose plant distribution is

not affected by the differential environments of the gap gradient, the population structure changes after human disturbances.

Interestingly, one gap showed similar juvenile density to the early successional forest (0.206 ind m^{-2}, gap D5, Table 1). This gap had a large reproductive tree of *C. floribundus* near the limits of the Brokaw area providing seeds for colonization of the gap (Fig. 2). Also, other gaps that had adjacent old gaps (A.M.Z Martini and R.A.F. Lima, unpublished data), with reproductive trees of the species, presented relatively higher juvenile density (for instance, gap G11, Table 1 and Fig. 2). The opposite response obtained in gap H9, which was not surrounded by any tree of *C. floribundus*, should be highlighted. These results indicate that rather than gap area or the suitable sites available for plant establishment, the source of seeds and barriers to seed arrival such as the short-distance seed dispersal mechanism seem to be determinant of the first-colonizer abundance in both forest types. As already mentioned above, this may also be one of the explanations for the absence of relation between gap area and plant density in the primary forest. Other barrier to the seed arrival in the open gaps might be the presence of large trees and dense tangle of lianas in the primary forest (Rodrigues 2005; Lima et al. 2008).

Plants of different life stages, namely, juvenile 1, juvenile 2 and reproductives of *C. floribundus* occurred spatially segregated in a 25 year-old secondary forest according to Danciguer (1996). In the primary forest analyzed here, trees were apparently segregated from juveniles but not from juveniles 2 while juveniles 2 were not spatially segregated from juveniles. In the early successional forest, juveniles occurred in the same transects of trees. The spatial structure of the species seems to be in accordance with the idea that there is a slow growth and an accumulation of juveniles in secondary forests, particularly in the early or mid successional stages, due to the unfavorable growth conditions for this size-class such

as the shading of tree crowns (Danciguer 1996; Goodale et al. 2012). Despite its relatively higher shade-tolerance and therefore the capability of surviving under more shaded environments, the species still requires high light conditions for recruitment to higher size-classes. Conversely, in the primary forest, where gaps containing juveniles usually do not present trees of *C. floribundus* or any other pioneer species, there are light and temperature conditions for rapid growth and thus, transition from juvenile to juvenile 2 must be facilitated. This accumulation of individuals in time in the early successional forest may indicate that juveniles came from several events of seed dispersal like in the secondary forest studied by Danciguer (1996). In addition, there might be an increase in number of migrants by seeds after colonization due to reduction of competition with grass and weeds that would result in higher juvenile density. Thus, the specific biotic and abiotic conditions of the early successional forest may favour survival of juveniles but hinder their growth and transition to the following size-class.

Likewise, competition and predation may affect juvenile density in a different way in the primary forest. In general, juveniles from the early successional forest showed phenotypic characteristics very different from the primary forest, such as less freshness and more damaged leaves, presence of re-sprouts, lower height and larger diameter at soil height (data not shown), which indicates more injuries and signs of recovery through re-sprouting. A high ability of re-sprouting in trees of *C. floribundus* as well as ramification in life-stage juvenile 1 (Danciguer 1996; Martini et al. 2008) might be an important factor to increase potential competitive and survival of the species. Thus, it is likely that individuals in new highly human disturbed environments use this ability to maintain growth even with very slow rates and survive in the most critical life stages. In the primary forest, on the other hand, the plants seem not to have this opportunity of regrowth and formation of a

"seedling bank". Besides the already mentioned high light availability in the gaps, other site-specific factors like seedling predation/herbivory (Uhl et al. 1991) and resource competition with lianas in the gaps (Schnitzer et al. 2005; Toledo-Aceves and Swaine 2008) may affect survival and growth preventing the formation of a seedling bank. This statement is based on the high density of lianas found in the gaps of the permanent plot of CES (Lima et al. 2008) and our observations of juvenile mortality.

The results showed that population structure of *C. floribundus*, a long-lived pioneer tree species with rapid growth varied between areas with contrasting levels of human disturbance but not with the level of natural disturbance. It seems that the main ecological processes that determine the pioneer species distribution in natural and human modified forests are the same, that is, responses to light, seed dispersal (limited), and colonization. However, different factors or conditions generated by those contrasting levels and types of disturbances seem to affect the way that the processes act. Hence, while the relative shade-tolerance allows *C. floribundus* to have a broader distribution in the gap-gradient of the primary forest, the unavailability of favorable sites for juvenile growth may contribute to high juvenile density in the early successional forest. Likewise, the colonization of a large open area created by intensive land use and with possible barriers to plant establishment associated to a limited seed dispersal decreases the density of trees in the early successional forest, whereas the distance from the source of seeds and barriers to seed dispersal decreases the density of juveniles in the primary forest.

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Tables

Table 1 Plant density of *Croton floribundus* Spreng. (ind m^{-2}) in sixteen canopy gaps in the permanent plot at Caetetus Ecological Station (CES). Age 3 = before 2002, Age 2= after 2002, Age 1 = after 2002, but more recent than age 2 (see more details in the text)

Gap	Age	Brokaw area (m^2)	Plant density (ind m^{-2})			
			Seedlings	Juveniles	Juveniles 2	Trees
D12	3	353	0	0.003	0.034	0.031
D5[a]	2	107	0	0.206	0.009	0
D7[a]	1	396	0.005	0.015	0.045	0
E11	3	81	0	0.062	0.074	0
F11	3	251	0	0.004	0.012	0.024
F7[a]	2	236	0.064	0.042	0.034	0.008
F9[a]	2	306	0	0.033	0.010	0
G10	1	23	0	0.086	0.043	0
G11	3	228	0	0.066	0.018	0
G7[a]	1	437	0	0.007	0.048	0.005
H9	2	80	0	0	0	0
H10	2	26	0	0.038	0.038	0
N8	2	129	0	0.039	0.218	0
P10	3	99	0	0	0	0.030
P11[a]	1	182	0.011	0	0	0
P3/P4[a]	2	645	0.059	0.050	0.022	0.005

[a] Complex gaps (gaps with recurrent tree disturbances)

Figure legends

Fig. 1 Location of the study sites: the permanent plot in the primary forest at Caetetus Ecological Station (CES plot) and the early successional forest at Torrão de Ouro Farm. Satellite imagery © 2012 MapLink/Tele Atlas, GeoEye Image, via Google Earth

Fig. 2 Map of the permanent subplots (20 x 20 m) at Caetetus Ecological Station (CES) showing the subplots (in yellow) where the 100 canopy gaps recorded by Lima et al. (2008) and A.M.Z Martini and R.A.F. Lima (unpublished data) were located and the trees (DBH ≥ 4.78 cm) of *Croton floribundus* Spreng. (red circles) censused by Rodrigues (2005)

Fig. 3 Scatter diagram of plant density (all size classes) of *Croton floribundus* Spreng. (ind m^{-2}) as a function of Brokaw gap size (m^2) (a) and gap age class (b) in sixteen canopy gaps in the permanent plot at Caetetus Ecological Station (CES). Age 3 = before 2002, Age 2= after 2002, Age 1 = after 2002, but more recent than age 2 (see more details in the text)

Fig. 4 Scatter diagram of tree density (DBH ≥ 4.78 cm) of *Croton floribundus* Spreng. (ind m^{-2}) as a function of gap age class in sixteen canopy gaps in the permanent plot at Caetetus Ecological Station (CES). Age 3 = before 2002, Age 2= after 2002, Age 1 = after 2002, but more recent than age 2 (see more details in the text)

Fig. 5 Box plot of tree DBH of *Croton floribundus* Spreng. in the permanent plot at Caetetus Ecological Station (CES) censused in 2005 (Rodrigues 2005) and 2010 (T.E. Barreto, unpublished data). The length of each box shows the range within which the central 50% of the values fall, with the box edges at the first and third quartiles. * = outliers

Fig. 6 Logistic regression of trees in bloom on DBH (cm) for *Croton floribundus* Spreng. in the permanent plot at Caetetus Ecological Station (CES)

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SP-331
N
CES plot
Torrão de Ouro Farm
© 2013 MapLink
Image © 2013 DigitalGlobe
Google earth
2006
Data das imagens: 3/4/2013 22°24'24.45"S 49°42'13.56"O elev 583 m altitude do ponto de visão 11.45 km

Fig. 1

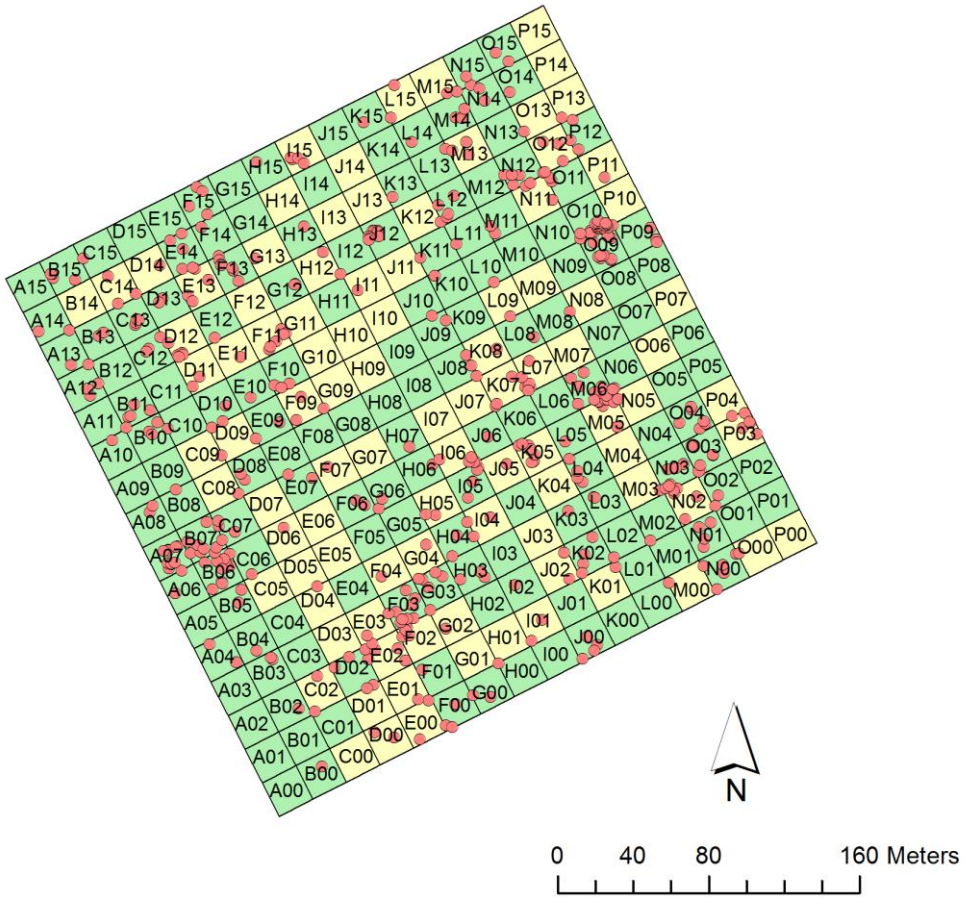

Fig. 2

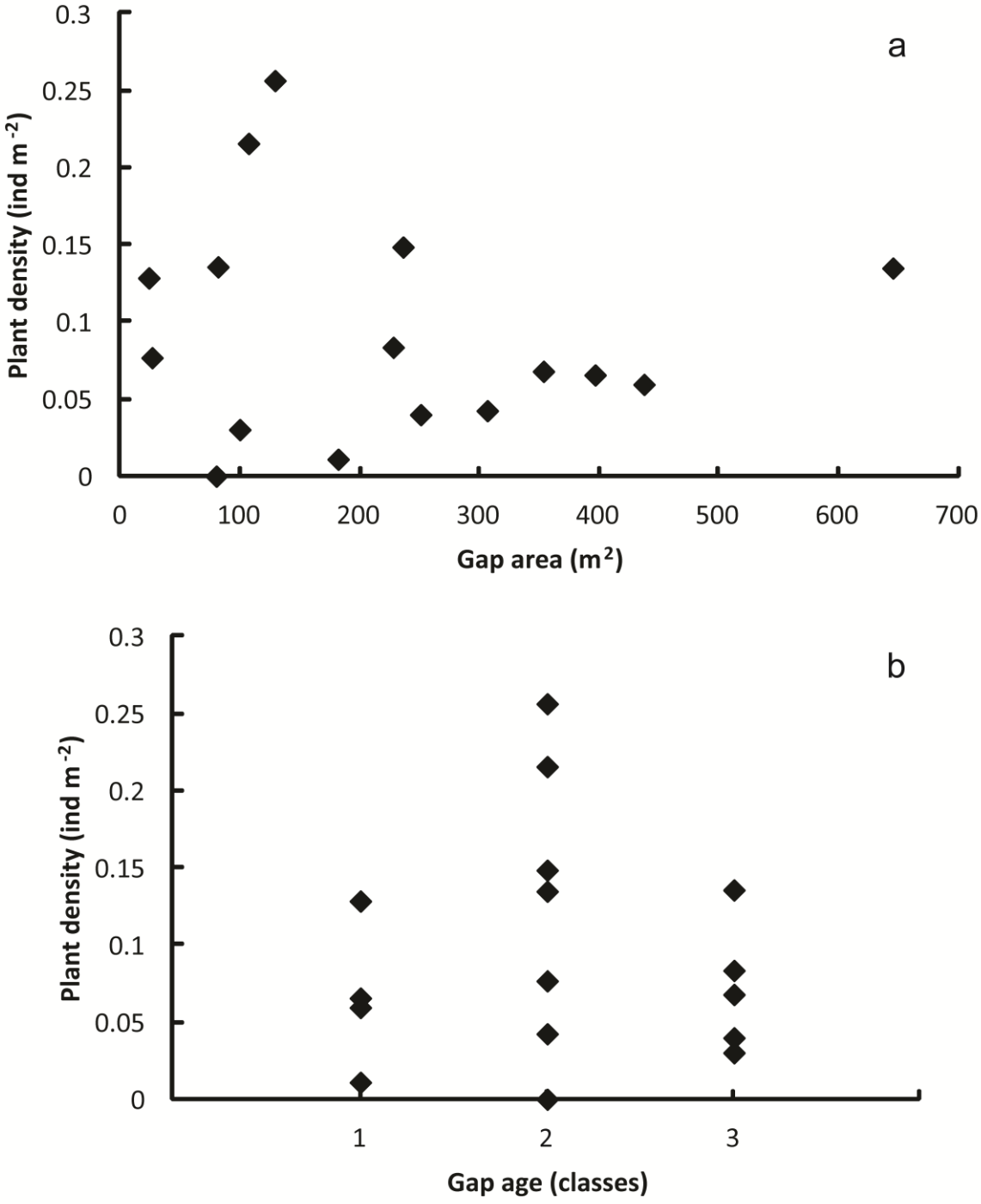
a
Plant density (ind m-2)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
100
200
300
400
500
600
700
Gap area (m2)
b
Plant density (ind m-2)
0.3
0.25
0.2
0.15
0.1
0.05
0
1
2
3
Gap age (classes)

Fig. 3

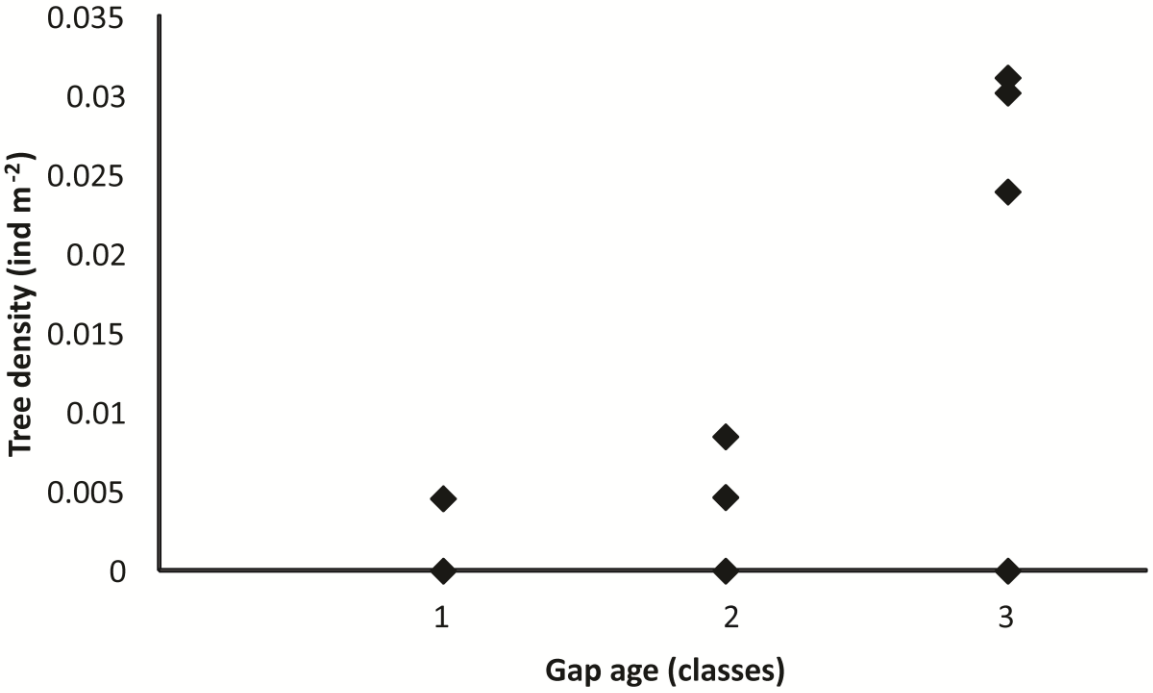
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
Tree density (ind m-2)
1
2
3
Gap age (classes)

Fig. 4

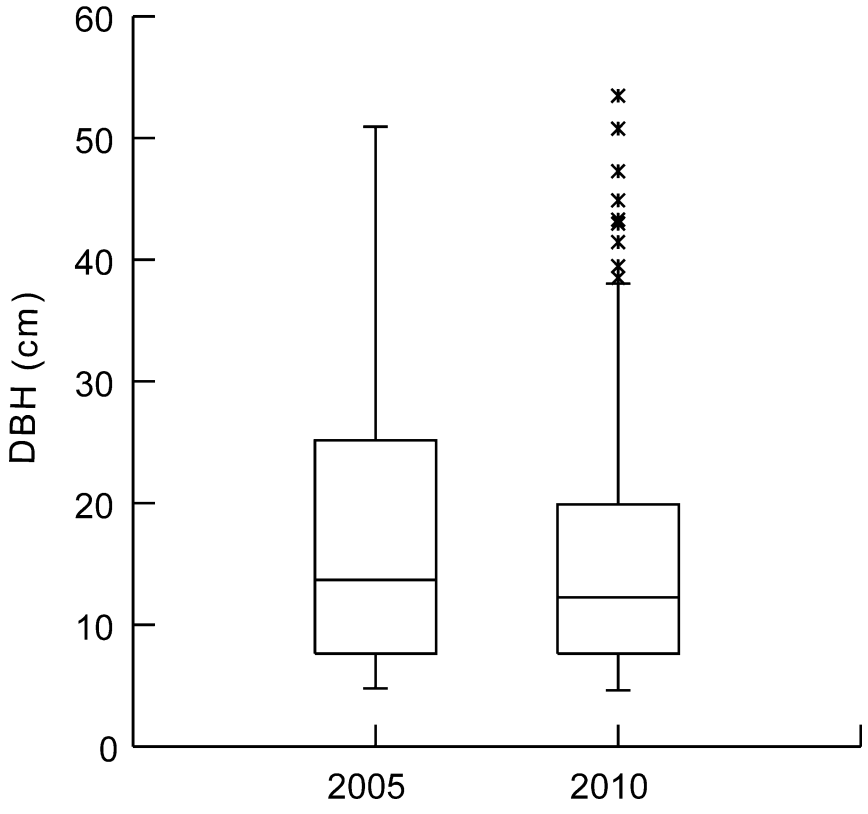
60
50
40
30
20
10
0
DBH (cm)
2005
2010

Fig. 5

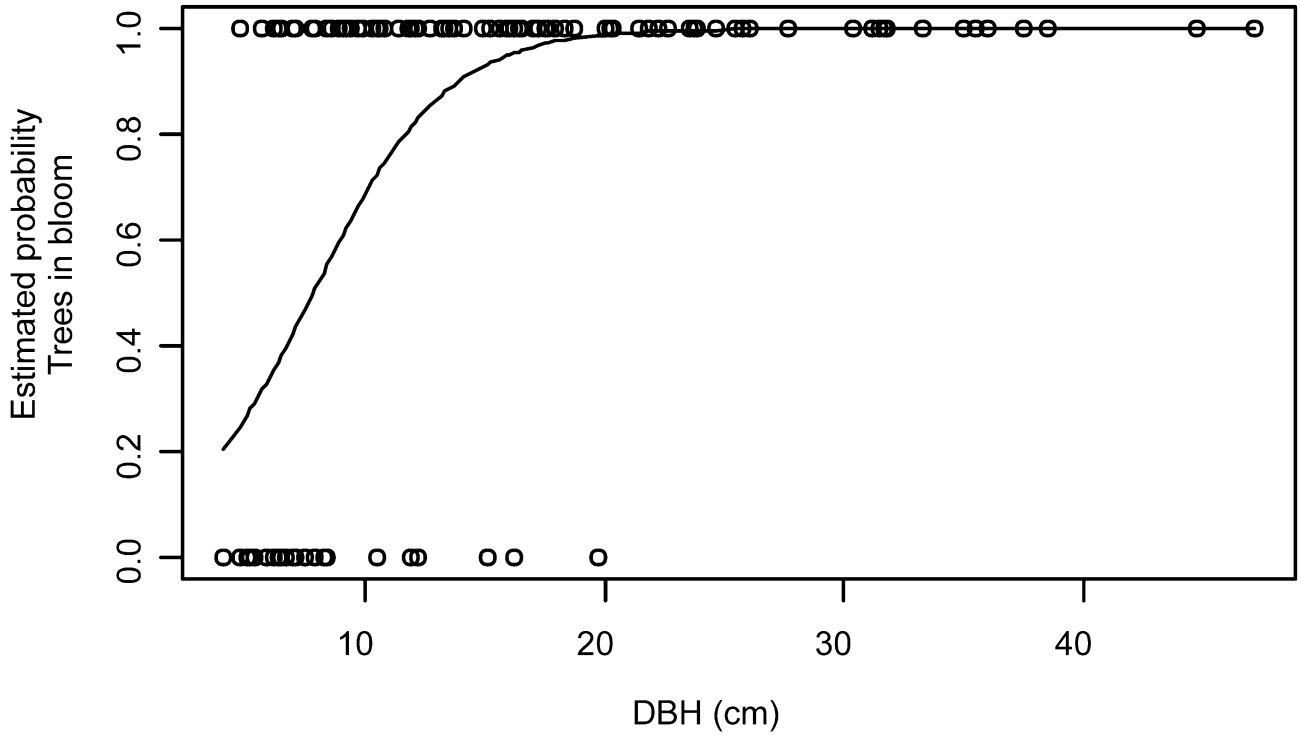
Estimated probability
Trees in bloom
0.0
0.2
0.4
0.6
0.8
1.0
10
20
30
40
DBH (cm)

Fig. 6

CHAPTER II

Amplificação e caracterização de marcadores microssatélites cloroplastidiais universais em *Croton floribundus* Spreng. (Euphorbiaceae) em uma floresta primária do Estado de São Paulo.

RESUMO

A avaliação da diversidade e estrutura genética de populações de plantas por meio de marcadores cloroplastidiais possibilita a realização de inferências a respeito do fluxo gênico por sementes bem como facilita a compreensão do processo de colonização e extinção. O presente trabalho teve como objetivo testar iniciadores microssatélites cloroplastidiais considerados universais na espécie arbórea pioneira *Croton floribundus* Spreng. e caracterizá-los por meio do estudo da diversidade e estrutura genética dentro e entre populações da espécie em uma floresta primária. Dez pares de iniciadores microssatélites cloroplastidiais (cpSSR) considerados universais foram testados sob diferentes condições de adstringência da reação de PCR em *C. floribundus*. Os pares de iniciadores ccmp01, ccmp08 e ccmp09 não amplificaram em *C. floribundus.* O par de iniciadores ccmp06 apresentou amplificações inespecíficas sob todas as condições de PCR testadas. Os locos ccmp02, ccmp03, ccmp04, ccmp05, ccmp07 e ccmp10 amplificaram fragmentos de tamanhos esperados em temperaturas de anelamento que variaram de 55^{o}C a 58^{o}C e foram marcados com fluorescência (6-FAM e 6-HEX) para genotipagem de 64 indivíduos de *C. floribundus* distribuídos em quatro clareiras. Apenas um haplótipo foi detectado, mostrando a ausência de variabilidade e estrutura genética cloroplastidial nas populações avaliadas. O monomorfismo encontrado pode indicar uma origem comum destas populações e/ou que seus colonizadores vieram de poucas populações próximas e bastante semelhantes geneticamente, já que a autocoria associada à presença de grandes árvores e densos emaranhados de lianas pode restringir as distâncias de dispersão de sementes da espécie na floresta primária. Polimorfismos são esperados em escalas geográficas mais amplas.

Palavras-chave: clareiras, DNA cloroplastidial, florestas tropicais, estrutura genética, microssatélites.

ABSTRACT

The study of genetic differentiation among plant populations using chloroplast markers allows us to infer the patterns of gene flow by seeds as well as to understand the process of colonization and extinction. The aim of this study was to test and characterize the amplification of universal chloroplast microsatellite primers in the pioneer tree *Croton floribundus* Spreng by means of the study of the genetic diversity and structure within and among populations of the species in a primary forest. Ten universal chloroplast microsatellite (cpSSR) primer pairs were tested using different stringencies of PCR conditions for *C. floribundus*. The primer pairs ccmp01, ccmp08, and ccmp09 did not amplify in *C. floribundus.* The primer pair ccmp06 showed unspecified amplification products in all PCR conditions tested. The loci ccmp02, ccmp03, ccmp04, ccmp05, ccmp07, and ccmp10 amplified the expected PCR products at annealing temperatures ranging from 55^{o}C to 58^{o}C. These loci had the 5' end forward primers fluorescent-dye labeled (6-FAM and 6-HEX) in order to genotype the 64 individuals sampled in four gaps. Only one haplotype was observed showing an absence of genetic variability within and among populations. The monomorphism found may indicate that populations present a common origin and/or their colonizers were drawn from a few near and genetically similar populations as a result of the limited seed dispersal of the species generated by autochory and barriers to seed dispersal in the primary forest. Polymorphisms are expected at larger spatial scales.

Key words: chloroplast DNA, gaps, genetic structure, microsatellites, tropical forests.

INTRODUÇÃO

As florestas tropicais brasileiras vêm sofrendo um intenso processo de desmatamento e fragmentação antes mesmo do conhecimento desses ecossistemas (Leitão-Filho 1987; Dean 1996; Ribeiro et al. 2009). De modo geral, a conservação da diversidade biológica presente nesses ecossistemas está diretamente ligada à conservação da variabilidade genética. Isto porque é através da variação genética disponível que os organismos podem responder às possíveis mudanças ambientais, mantendo sua capacidade de adaptação e garantindo sua sobrevivência e reprodução ao longo do tempo (Koskela & Amaral 2002; Namkoong et al. 2002, Hedrick 2005). A diversidade genética é introduzida continuamente nas populações por mutação e fluxo gênico e pode ser perdida por deriva genética, endocruzamentos e pela maior parte dos tipos de seleção natural (Nei 1987; Cole 2003).

O fluxo gênico promove a conectividade genética transferindo a variabilidade genética entre as populações (Hamrick & Nason 2000). De acordo com Ennos (1994), o fluxo gênico entre populações de plantas pode ocorrer de duas maneiras. A primeira envolve a dispersão do pólen de uma população diferente, a fecundação de um óvulo por este pólen e, finalmente, o estabelecimento da semente. O fluxo de genes pode ocorrer também pela dispersão das sementes, e o sucesso no estabelecimento das sementes dispersas dentro de uma nova população.

Os mecanismos de dispersão que se incluem no fluxo gênico, tanto via pólen quanto via sementes, são muito importantes, pois podem modelar a composição e a estrutura genética das populações de plantas (Dow & Ashley 1998). Comumente, polinizadores e agentes dispersores de sementes que apresentem efetiva dispersão a longas distâncias diminuem a probabilidade de ocorrer diferenciação geográfica entre

populações, enquanto a dispersão em distâncias restritas tem efeito contrário, promovendo a estruturação genética populacional (Loveless & Hamrick 1984). Deste modo, espécies arbóreas polinizadas pelo vento, ou seja, cujo pólen é disperso a longas distâncias, tendem a ter maiores taxas de fluxo gênico e menor diferenciação genética que espécies polinizadas por insetos ou outros animais, por exemplo. Já espécies autocóricas tendem a apresentar uma dispersão de sementes limitada espacialmente, resultando em uma maior diferenciação genética entre as populações (Loveless & Hamrick 1984; Hamrick & Nason 2000). Entretanto, algumas árvores tropicais polinizadas por insetos apresentaram distâncias de migração de pólen de várias centenas de metros, apresentando também altas taxas de fluxo gênico, como as espécies polinizadas pelo vento (Hamrick & Nason 2000; Silva et al. 2008).

Entre os diversos marcadores moleculares disponíveis atualmente, os marcadores microssatélites ou *Simple Sequence Repeats* (SSR) são os mais viáveis para a avaliação da diversidade e estrutura genética, pois são codominantes, apresentam alto poliformismo e são abundantes nos genomas das plantas (Ferreira & Grattapaglia 1998). De acordo com Lemes et al. (2003), os marcadores SSR têm sido cada vez mais utilizados como um instrumento eficaz para a compreensão da estrutura genética de populações, fluxo gênico, grau de parentesco, viabilidade de populações, além de permitir a quantificação dos efeitos da fragmentação de hábitats e estabelecer estratégias para a conservação de espécies. Os níveis de fluxo gênico e a proporção de fluxo gênico de pólen e sementes têm sido estimados indiretamente baseados na distribuição de diversidade genética entre as populações (F_{ST}-Wright 1931, 1951; G_{ST}-Nei 1973) por meio de marcadores microssatélites nucleares e cloroplastidiais em espécies arbóreas do Cerrado (Gaiotto et al. 2003; Martins 2005; Moreno et al. 2009),

da Floresta Ombrófila (Lira et al. 2003; Azevedo et al. 2008, Born et al. 2008) e da Floresta Estacional Semidecidual (Monteiro et al. 2009). Na maioria destes estudos foram utilizados os microssatélites cloroplastidiais universais, ou seja, iniciadores cpSSR com sequências conservadas que foram desenvolvidos para amplificar em diferentes famílias de dicotiledôneas (Weising & Gardner 1999).

Esse tipo de análise é possível, porque o DNA nuclear é de herança biparental, transmitido através de semente e pólen, enquanto o DNA das organelas, mitocondrial (mtDNA) ou cloroplastidial (cpDNA) geralmente é de herança uniparental (maternal) em angiospermas, sendo transmitido através de sementes (Parker et al. 1998). Portanto, comparando-se os diferentes padrões dos marcadores nucleares e cpDNA, há a possibilidade de inferir os níveis relativos de fluxo gênico por pólen e sementes (Ennos 1994; Ouborg et al. 1999). No entanto, a estimativa de fluxo gênico por meio indireto é baseada em algumas premissas que nem sempre são encontradas nas populações naturais. Entre as principais está o equilíbrio entre deriva genética e fluxo gênico, que não se verifica especialmente em populações que sofrem recorrentes extinções e colonizações (Whitlock & McCauley 1999, Ouborg et al 1999), como nas espécies arbóreas pioneiras. Para estas espécies, a obtenção da diferenciação genética entre populações usando marcadores cloroplastidiais e nucleares pode ser suficiente e muito informativa para a compreensão de alguns padrões e processos populacionais tais como o processo de colonização e extinção e para inferências dos padrões de dispersão de pólen e sementes juntamente com análises de autocorrelação espacial (McCauley 1995; Whitlock & McCauley 1999; Hamrick & Trapnell 2011).

A espécie arbórea *Croton floribundus* Spreng., conhecida como capixingui, é considerada uma espécie pioneira, ou seja, sua história de vida e estrutura populacional

são afetadas pela dinâmica de clareiras (Lorenzi 1992; Gandolfi et al. 1995; Rodrigues 1995). Deste modo, é uma árvore de crescimento rápido e intolerante à sombra que ocorre abundantemente nas clareiras de remanescentes primários da Floresta Estacional Semidecidual (Lorenzi 1992; Gandolfi et al. 1995; Rodrigues 1999; Rodrigues 2005), e cuja dinâmica demográfica assemelha-se a de uma metapopulação, ou seja, as populações persistem em uma escala regional em consequência do processo local de colonização e extinção das clareiras (Freckleton & Watkinson 2002).

Espécies pioneiras polinizadas por insetos têm mostrado baixos níveis de diferenciação genética entre populações e altas taxas de fluxo gênico (Litrico et al. 2005; Born et al. 2008). Entretanto, estas espécies apresentam também dispersão de sementes a longas distâncias por vento ou pássaros, o que pode estar influenciando os resultados encontrados. Já a espécie *C. floribundus* é polinizada por insetos e vento (Passos 1995), mas as sementes são dispersas a curtas distâncias pela abertura explosiva dos frutos (autocoria) (Lorenzi 1992; Stamp & Lucas 1983, Passos & Ferreira 1996). Neste caso, o estudo da diversidade e estrutura genética utilizando marcadores cloroplastidiais seria útil para elucidar como o mecanismo de dispersão de sementes por autocoria pode afetar a distribuição da variação genética de uma espécie pioneira. Do mesmo modo, a análise de marcadores cloroplastidiais transmitidos exclusivamente via sementes em populações de *C. floribundus* ocorrendo em uma área de floresta conservada (primária) pode contribuir para o entendimento do efeito do processo de colonização e extinção gerado pela dinâmica de clareiras sobre suas características genéticas.

Neste contexto, o presente trabalho teve como objetivos:

1) Testar iniciadores microssatélites cloroplastidiais considerados universais na espécie pioneira arbórea *C. floribundus* para uso em futuros estudos genéticos da espécie.

2) Caracterizar tais marcadores microssatélites cloroplastidiais em *C. floribundus*, avaliando a diversidade e estrutura genética dentro e entre populações da espécie em uma floresta primária.

MATERIAIS E MÉTODOS

Local de estudo

O estudo foi realizado dentro de uma parcela permanente de 10,24 ha na Estação Ecológica dos Caetetus (EEC) (Rodrigues 2005). A EEC está localizada no município de Gália e Alvilândia, Estado de São Paulo, sudeste do Brasil (22°20' - 22°30'S; 49°40' - 49°45'O). O clima regional corresponde ao tipo Cwa, de acordo com a classificação de Köppen, ou seja, mesotérmico com um inverno seco (Rodrigues 2005; Tabanez et al. 2005). As médias anuais de precipitação e temperatura são de 1.431 $mm.ano^{-1}$ e 21,5º C, respectivamente. A altitude varia de 520 a 680 m (Tabanez et al. 2005). Os solos predominantes na região da EEC são os argissolos e, em áreas com declives inferiores a 5%, os latossolos (Tabanez et al. 2005; Rodrigues 2005).

A EEC é um remanescente da Floresta Estacional Semidecidual (IBGE 1992) com 2.178,84 ha, que corresponde à Floresta Subtropical Úmida, de acordo com a classificação de Holdridge (Holdridge 1967). A Estação Ecológica dos Caetetus foi criada em 1987 e preserva uma das maiores áreas contínuas representativas dessa formação florestal, que revestia o planalto ocidental paulista (Tabanez et al. 2005). A estação representa uma base para o conhecimento da estrutura e do funcionamento da

comunidade florestal original, além de se destacar pelo excelente estado de conservação da maior parte de sua área (Tabanez et al. 2005). De acordo com relatos históricos da região, que datam do início do século 20, não há registros de perturbações antrópicas no núcleo do parque, onde foi estabelecida a parcela permanente de 10,24 ha. Por outro lado, Lima et al. (2008) e Martini et al. (2008) mostraram que a floresta nesta área é muito dinâmica, apresentando clareiras maiores que em outras florestas tropicais, além de alta densidade de clareiras e alta percentagem de área total ocupada por clareiras.

Espécie estudada

A espécie arbórea *Croton floribundus* Spreng. (Euphorbiaceae), conhecida popularmente como capixingui, é classificada como uma espécie monóica, com dicogamia (Passos 1995), crescimento rápido, intolerância ao sombreamento e é muito comum em clareiras das florestas semidecíduas (Rodrigues 1999). É considerada uma espécie pioneira (Lorenzi 1992; Gandolfi et al. 1995; Rodrigues 1995), mas apresenta algumas características incomuns deste grupo, tais como sementes grandes (Válio & Scarpa 2001) e ausência de banco de sementes e de reprodução assexual (Passos 1995; Danciguer 1996). A dispersão de sementes é realizada por autocoria através da deiscência explosiva dos frutos (Lorenzi 1992). Segundo Passos (1995), *C. floribundus* é polinizada principalmente por moscas da família Syrphidae e por anemofilia.

Plantas de *C. floribundus* da classe de tamanho jovem (Capítulo I) foram selecionadas como objeto deste estudo por serem facilmente encontrados e distinguíveis dentro de cada clareira. Indivíduos jovens caracterizam-se por não terem cotilédones, embora possam ter alguma estrutura embrionária. Possuem altura

variando entre 5 e 37 cm e diâmetro a altura do solo (DAS) entre 0,05 e 0,40 cm (Danciguer 1996) (Figura 1).

Delineamento amostral

As plantas avaliadas foram amostradas em quatro clareiras (populações) encontradas na parcela permanente da EEC, conforme Lima et al (2008) e Martini, AMZ & Lima, RAF (dados não publicados) (Figura 2). As clareiras foram denominadas de acordo com a subparcela onde se encontram. Assim, foram amostrados 16 indivíduos na clareira C5; 18 indivíduos na clareira E13; 16 indivíduos na clareira K8; e 14 indivíduos na clareira M5 (Figura 2). No total, 64 indivíduos foram amostrados.

Material vegetal

Foram utilizadas folhas jovens de cada planta avaliada, as quais foram armazenadas separadamente em freezer a –80ºC e, posteriormente, maceradas com nitrogênio líquido em almofariz, para extração de DNA.

Extração e quantificação de DNA

O DNA total (nuclear e cloroplastidial) foi extraído de folhas jovens de acordo com Doyle & Doyle (1990), utilizando CTAB como detergente. A quantificação do DNA total das amostras foi estimada pela intensidade de fluorescência emitida pelo corante *Sybr safe* (Invitrogen Corporation) por eletroforese em géis de agarose a 0,8%. Essa intensidade foi comparada a de padrões com concentrações específicas e conhecidas

(DNA do Fago λ - lambda). Após a quantificação, as amostras de DNA foram diluídas para uma concentração final de 20ng/μL.

Marcadores microssatélites cloroplastidiais (cpSSR)

Foram utilizados os marcadores cloroplastidias considerados universais desenvolvidos para dicotiledôneas por Weising & Gardner (1999) (Tabela 1). Os mesmos foram testados e otimizados em *C. floribundus.*

Amplificação dos locos cpSSR

Para amplificação dos locos microssatélites cloroplastidiais, foram inicialmente testadas as mesmas condições de reação utilizadas para os marcadores nucleares que estavam sendo desenvolvidos para a espécie: 40ng de DNA total, 1x tampão, 0,1mM dNTP, 2mM $MgCl_2$, 10pmoles de cada iniciador, 1 unidade de Taq DNA polimerase para um volume final de 25μL (Capítulo III). O programa de amplificação foi constituído por um ciclo inicial de 5 minutos de desnaturação (95ºC); seguido por 30 ciclos de 1 minuto de desnaturação (95ºC), 1 minuto de anelamento (55ºC) e 1 minuto de extensão (72ºC); e um ciclo final de 10 minutos de extensão (72ºC). Variações na adstringência da reação de PCR foram testadas com o objetivo de otimizar a amplificação dos locos (temperatura e $MgCl_2$). Assim, para os iniciadores que apresentaram bandas inespecíficas ou má qualidade de amplificação do produto específico foram testados gradientes de temperatura entre 60ºC e 50ºC, concentrações de $MgCl_2$ de 1,5mM e 1,0mM, bem como as condições de reação originais de Weising & Gardner (1999)

descritas a seguir: 20ng de DNA total, 1x tampão, 0,2mM dNTP, 2,5mM $MgCl_2$, 5pmoles de cada iniciador, 1 unidade de Taq DNA polimerase para um volume final de 10µL.

Visualização da amplificação

Todas as reações de PCR foram analisadas em eletroforese de gel de agarose a 2% e TBE 1x para verificação da presença de produtos de amplificação. Os produtos de PCR foram visualizados por meio do corante fluorescente *blue green loading dye I* (LGC Biotecnologia). Após a otimização dos pares de iniciadores, os produtos de amplificação dos locos foram visualizados em géis de poliacrilamida 6% não desnaturantes corados com nitrato de prata para confirmação dos resultados.

Visualização de polimorfismo

Os fragmentos de microssatélites cloroplastidiais foram visualizados por meio do sistema de eletroforese capilar em um sequenciador automático 3730 DNA Analyzer (Applied Biosystems, Inc.), utilizando iniciadores marcados com fluorescência e marcadores internos de tamanhos moleculares conhecidos (GENESCAN-500 ROX, Applied Biosystems, Inc.). Para isso, os iniciadores otimizados foram marcados com fluorocromos específicos (6-FAM e 6-HEX) de acordo com o tamanho esperado de cada loco (Tabela 1): 6-FAM para os locos com produto esperado maior ou igual a 126 pb e 6-HEX para os locos com produto esperado menor que 126pb. Tal procedimento permitiu a genotipagem *multiplex*, ou seja, a diferenciação de dois locos cpSSR em uma única corrida. A combinação de locos para aplicação em *multiplex* foi avaliada concomitantemente aos testes com diferentes concentrações de iniciadores (5 e 10pmoles) e volume de reação a ser injetado no sistema de eletroforese capilar (1,0 e

1,5 µL). As seguintes combinações de iniciadores foram testadas: ccmp02 (6-FAM) e ccmp3 (6-HEX), ccmp4 (6-FAM) e ccmp10 (6-HEX), ccmp7 (6-FAM) e ccmp5 (6-HEX). Alíquotas das reações foram visualizadas em gel de agarose 2% para verificar a qualidade de amplificação, antes do envio para a automatização da eletroforese. As amostras foram preparadas para genotipagem utilizando-se 9µL de formamida, 0,5µL do padrão de peso molecular (GENESCAN-500 ROX) e o volume de produtos de PCR mais adequado para genotipagem *multiplex*.

Análise de Dados

Amplificação dos microssatélites cloroplastidiais universais

Os géis de agarose em eletroforese para amplificação dos locos cpSSR em *C. floribundus* foram analisados quanto à qualidade: 1) foi observado se os locos foram amplificados; 2) quanto ao tamanho do produto, se o mesmo apresentou variações de tamanho dentro do esperado pela literatura (Weising & Gardner 1999); 3) quanto ao aparecimento de bandas inespecíficas, sugerindo necessidade de otimização das condições de reação. Da mesma forma, foi avaliada a amplificação com os iniciadores marcados com fluorescência, ou seja, os resultados da eletroforese no sequenciador automático 3730 DNA Analyzer foram analisados principalmente com relação ao tamanho e intensidade dos picos SSR.

Visualização de polimorfismo

Os fragmentos cpSSR (alelos) foram analisados após eletroforese, utilizando o programa GeneMapper v. 3.7 (Applied Biosystems, Inc.).

Diversidade e estrutura genética

Cada combinação alélica única entre todos os locos cpSSR analisados foi considerada um haplótipo. A partir desta definição avaliou-se o número de haplótipos presente nas populações.

RESULTADOS

Amplificação dos locos microssatélites cloroplastidiais

Dos dez marcadores cloroplastidiais desenvolvidos por Weising & Gardner (1999) (Tabela 1) e testados em *C. floribundus*, seis apresentaram excelente amplificação sob as mesmas condições de reação utilizadas para os marcadores nucleares, com variações na temperatura de anelamento (Figura 3). Assim, para os pares de iniciadores ccmp2, ccmp3, ccmp5, ccmp7, e ccmp10, que produziram bandas intensas, no tamanho esperado e sem amplificações inespecíficas, as condições ótimas de reação foram as seguintes: 40ng de DNA total, 1x tampão, 0,1mM dNTP, 2mM $MgCl_2$, 10pmoles de cada iniciador, 1 unidade de Taq DNA polimerase para um volume final de 25µL. O programa de amplificação foi constituído por um ciclo inicial de 5 minutos de desnaturação (95ºC); seguido por 30 ciclos de 1 minuto de desnaturação (95ºC), 1 minuto de anelamento (55ºC) e 1 minuto de extensão (72ºC); e um ciclo final de 10 minutos de extensão (72ºC). Para o loco ccmp4, foi necessário aumentar a adstringência da reação, sendo utilizada a temperatura de anelamento de 58ºC.

Os iniciadores ccmp1, ccmp8 e ccmp9 não amplificaram o produto esperado em *C. floribundus* sob nenhuma das condições testadas. O par de iniciadores ccmp6 produziu bandas inespecíficas e/ou má qualidade do produto esperado, não sendo encontrado um balanço adequado das condições de PCR para a amplificação do loco.

Portanto, dos dez iniciadores cpSSR avaliados, seis mostraram amplificação adequada e foram marcados com fluorescência (6-FAM e 6-HEX) para utilização na genotipagem de *C. floribundus*. Estes resultados foram confirmados pela visualização dos produtos amplificados em géis de poliacrilamida não desnaturantes (Figura 4).

Visualização de polimorfismo

Todas as combinações de locos para genotipagem *multiplex* testadas apresentaram picos com intensidades suficientes para análise e sem interferência entre locos, utilizando-se 10 pmoles de iniciador e 1,0 µL de cada produto de PCR. A utilização de 1,0 µL de produto de PCR minimizou a produção de picos com intensidade além da escala ("*off-scale*") verificados com o volume de 1,5 µL. Para alguns locos, a leitura dos picos foi eficiente também utilizando-se 5 pmoles de iniciadores e 1,0 µL de produto de PCR. Porém, optou-se pela utilização de 10 pmoles em todos os locos para padronização do protocolo de amplificação. A genotipagem *multiplex* mostrou-se uma importante ferramenta, pois permitiu a diminuição da quantidade de corridas por meio da leitura adequada de dois locos cpSSR no mesmo capilar (Figura 5).

As reações de amplificação com os seis iniciadores marcados com fluorocromo nos 64 indivíduos em estudo foram visualizadas primeiramente em gel de agarose, apresentando bandas de boa qualidade, com tamanho esperado e sem amplificações inespecíficas (Figura 6, Figura 7a). Posteriormente foram obtidos os resultados da genotipagem por meio do sistema de eletroforese capilar (Figura 7), onde se constatou que todos os seis locos analisados foram monomórficos nas populações estudadas (Tabela 2).

Diversidade e estrutura genética

Foi observado somente um haplótipo nos 64 indivíduos amostrados, o que revelou a ausência de variabilidade e estrutura genética cloroplastidial nas populações avaliadas de *C. floribundus* na floresta primária.

DISCUSSÃO

De modo geral, os tamanhos dos alelos dos seis locos cloroplastidiais avaliados em *C. floribundus* estão dentro da faixa amplificada em outras espécies arbóreas como em *Casearia sylvestris* Sw. (Salicaceae) na Floresta Estacional Semidecidual (Monteiro et al. 2009), em *Solanum lycocarpum* St. Hil. (Solanaceae) no Cerrado (Martins 2005), e em *Manilkara huberi* (Ducke) A. Chev. (Sapotaceae) na Floresta Amazônica (Azevedo 2008). Isto indica que as regiões amplificadas sofreram pequenas variações de tamanho, ou seja, provavelmente poucas inserções/deleções de sequências curtas (1 a 10pb) ao logo do processo evolutivo nestas famílias (Palmer 1987), já que neste nível hierárquico, outros eventos mutacionais que não a variação no número de repetições dos cpSSR parecem ser responsáveis pelas diferenças nos tamanhos destas sequências (Weising & Gardner 1999). Além disso, a amplificação e pouca variação no tamanho das sequências em diferentes famílias de plantas confirma que esses locos, em especial as regiões flanqueadoras dos microssatélites, são bastante conservados nas plantas superiores (Weising & Gardner 1999). O loco ccmp2 foi o único que apresentou tamanho um pouco acima do esperado de acordo com a lista de espécies apresentada por Weising & Gardner (1999) (166 – 234pb). Embora mais rara, esta magnitude de variação no tamanho alélico dos cpSSR também pode ocorrer nas angiospermas (Weising & Gardner 1999), já tendo sido encontrado tamanho de alelo

similar para o loco ccmp2 (280pb) na espécie do Cerrado *Hymenaea stigonocarpa* Mart. ex Hayne (Leguminosae-Caesalpinoideae) (Moreno et al. 2009). Tais variações de tamanho entre as espécies podem ser resultado de inserções ou deleções de sequências longas de nucleotídeos (50 a 1200pb), um tipo de mutação menos frequente que a de sequências curtas, mas ainda comum no DNA cloroplastidial (Palmer 1987).

Já o nível de polimorfismo intraespecífico encontrado nos cpSSR é devido principalmente às mutações graduais dos SSR ou à variação do número das sequências repetidas, no caso, de mononucleotídeos A e T (Weising & Gardner 1999), pois estas são mais frequentes que outros tipos de mutações no DNA cloroplastidial (Provan et al. 1999). O acesso a tal variabilidade permite inferências sobre os processos populacionais. Assim, os monomorfismos e a baixa variação intraespecífica verificados nos microssatélites cloroplastidiais avaliados por Weising & Gardner (1999) em *Nicotiana tabacum* L. e por Moreno et al. (2009) em *Hymenaea stigonocarpa* indicaram efeito de gargalo e baixa taxa de fluxo gênico por sementes, nas respectivas espécies estudadas. Já Martins (2005) encontrou um alto número de alelos por loco nas populações de *Solanum lycocarpum*, evidenciando, após análise comparativa com os marcadores nucleares, um papel importante da migração de sementes no fluxo gênico da espécie.

No caso de *C. floribundus*, assumindo-se a herança maternal dos locos cloroplastidiais, a ausência de variabilidade entre e dentro populações na floresta primária, pode indicar que seus colonizadores tiveram uma origem comum, ou seja, vieram de uma única população próxima, e/ou vieram de poucas populações também próximas e bastante semelhantes geneticamente. Considerando-se que as populações

ou clareiras amostradas estão localizadas a curtas distâncias uma da outra, esta similaridade genética ou ausência de variabilidade pode ser o resultado da limitada dispersão de sementes da espécie gerada pela autocoria (Loveless & Hamrick 1984; Hamrick & Nason 2000). Além disso, a presença de grandes árvores e densos emaranhados de lianas (Rodrigues 2005; Lima et al. 2008) pode ser uma barreira para a chegada de sementes nas clareiras na floresta primária, reduzindo a diversidade haplotípica. Por outro lado, a diversidade cloroplastidial na floresta primária pode estar distribuída em uma escala espacial maior do que a avaliada neste estudo. Esta questão poderá ser esclarecida através de estudos que avaliem a diversidade genética cloroplastidial em um maior número de clareiras ou em populações abrangendo escalas geográficas mais amplas. Neste sentido, os marcadores microssatélites cloroplastidiais otimizados para *C. floribundus* devem ser testados em escalas mais amplas e, uma vez apresentando polimorfismos, podem ser usados juntamente com análises de marcadores nucleares para inferências sobre o processo de colonização e a dispersão de sementes na espécie.

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TABELAS

Tabela 1: Locos, sequências *foward* (F) e *reverse* (R) dos iniciadores, motivo e tamanho em pares de bases (pb) dos microssatélites cloroplastidiais universais observados em tabaco por Weising & Gardner (1999).

Locos	Sequência dos iniciadores (5' – 3')	Motivo	Tamanho em tabaco (pb)
ccmp1	F= 5 '-CAGGTAAACTTCTCAACGGA-3' R= 5' -CCGAAGTCAAAAGAGCGATT-3'	$(T)_{10}$	139
ccmp2	F= 5'-GATCCCGGACGTAATCCTG-3' R= 5'-ATCGTACCGAGGGTTCGAAT-3'	$(A)_{11}$	189
ccmp3	F= 5'-CAGACCAAAAGCTGACATAG-3' R= 5'-GTTTCATTCGGCTCCTTTAT-3'	$(T)_{11}$	112
ccmp4	F= 5'-AATGCTGAATCGAYGACCTA-3' R= 5'-CCAAAATATTBGGAGGACTCT-3'	$(T)_{13}$	126
ccmp5	F= 5'-TGTTCCAATATCTTCTTGTCATTT-3' R= 5'-AGGTTCCATCGGAACAATTAT-3'	$(C)_7(T)_{10}$ $(T)_5C(A)_{11}$	121
ccmp6	F= 5'-CGATGCATATGTAGAAAGCC-3' R= 5'-CATTACGTGCGACTATCTCC-3'	$(T)_5C(T)_{17}$	103
ccmp7	F= 5'-CAACATATACCACTGTCAAG-3' R= 5'-ACATCATTATTGTATACTCTTTC-3'	$(A)_{13}$	133
ccmp8	F= 5'-TTGGCTACTCTAACCTTCCC-3' R= 5'-TTCTTTCTTATTTCGCAGDGAA-3'	$(T)_6C(T)_{14}$	77
ccmp9	F= 5'-GGATTTGTACATATAGGACA-3' R= 5'-CTCAACTCTAAGAAATACTTG-3'	$(T)_{11}$	98
ccmp10	F= 5'-TTTTTTTTTAGTGAACGTGTCA-3' R= 5'-TTCGTCGDCGTAGTAAATAG-3'	$(T)_{14}$	103

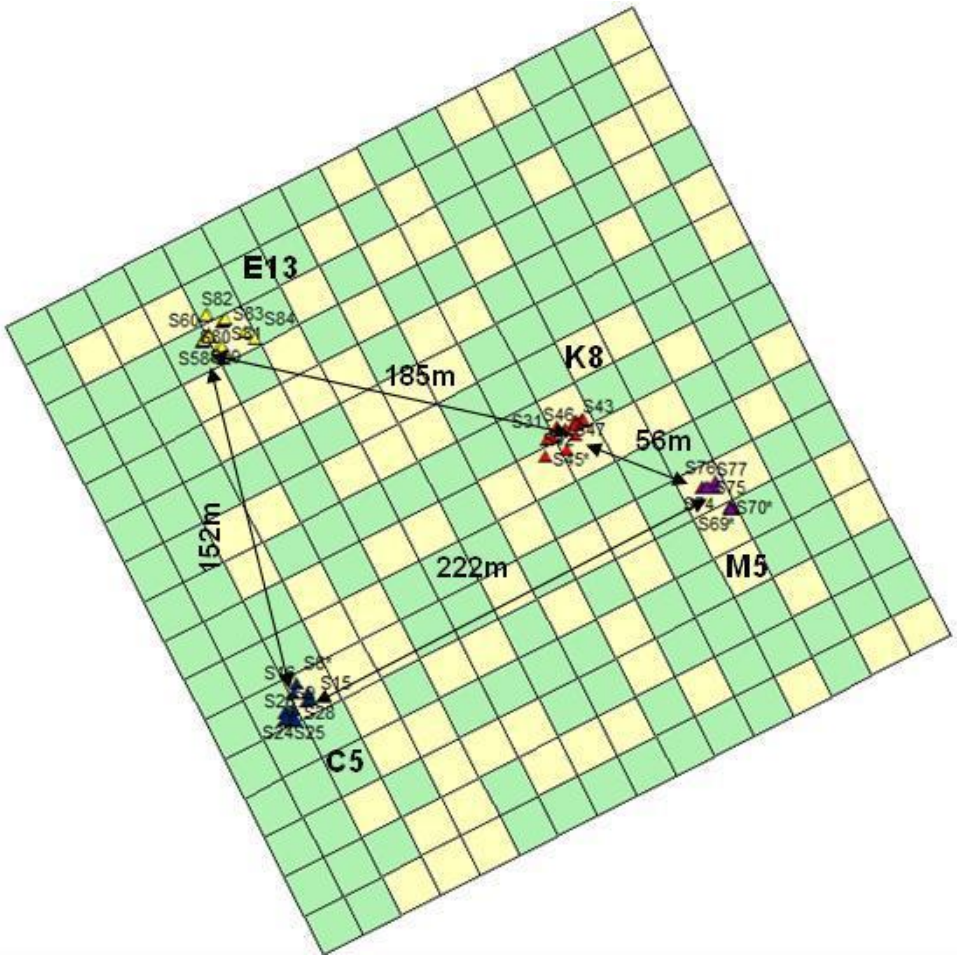
Tabela 2: Locos, temperatura de anelamento, sequências *foward* (F) e *reverse* (R) dos iniciadores, motivo, número de alelos e tamanho dos alelos em pares de bases (pb) dos microssatélites cloroplastidiais universais caracterizados em *Croton floribundus* Spreng.

Locos	T°(C)	Sequência dos iniciadores (5' – 3')*	Motivo*	Alelos	Tamanho dos alelos (pb)
ccmp2	55°	F= 5'-GATCCCGGACGTAATCCTG-3' R= 5'-ATCGTACCGAGGGTTCGAAT-3'	$(A)_{11}$	1	301
ccmp3	55°	F= 5'-CAGACCAAAAGCTGACATAG-3' R= 5'-GTTTCATTCGGCTCCTTTAT-3'	$(T)_{11}$	1	104
ccmp4	58°	F= 5'-AATGCTGAATCGAYGACCTA-3' R= 5'-CCAAAATATTBGGAGGACTCT-3'	$(T)_{13}$	1	112
ccmp5	55°	F= 5'-TGTTCCAATATCTTCTTGTCATTT-3' R= 5'-AGGTTCCATCGGAACAATTAT-3'	$(C)_7(T)_{10}$ $(T)_5C(A)_{11}$	1	109
ccmp7	55°	F= 5'-CAACATATACCACTGTCAAG-3' R= 5'-ACATCATTATTGTATACTCTTTC-3'	$(A)_{13}$	1	130
ccmp10	55°	F= 5'-TTTTTTTTTAGTGAACGTGTCA-3' R= 5'-TTCGTCGDCGTAGTAAATAG-3'	$(T)_{14}$	1	134

* De acordo com o observado em tabaco por Weising & Gardner (1999)

FIGURAS

Figura 1: Indivíduo de *Croton floribundus* Spreng. da classe de tamanho jovem amostrado em uma clareira na Estação Ecológica dos Caetetus.



Legenda:

- indivíduos jovens da clareira C5.
- indivíduos jovens da clareira E13.
- indivíduos jovens da clareira K8.
- indivíduos jovens da clareira M5.

Figura 2: Distribuição dos indivíduos jovens amostrados de *Croton floribundus* Spreng. nas subparcelas (20 x 20 m) permanentes da Estação Ecológica dos Caetetus. Escala 1:2.839. Subparcelas em amarelo indicam as subparcelas onde foram encontradas as 100 clareiras avaliadas por Lima et al (2008) e Martini, AMZ & Lima, RAF (dados não publicados).

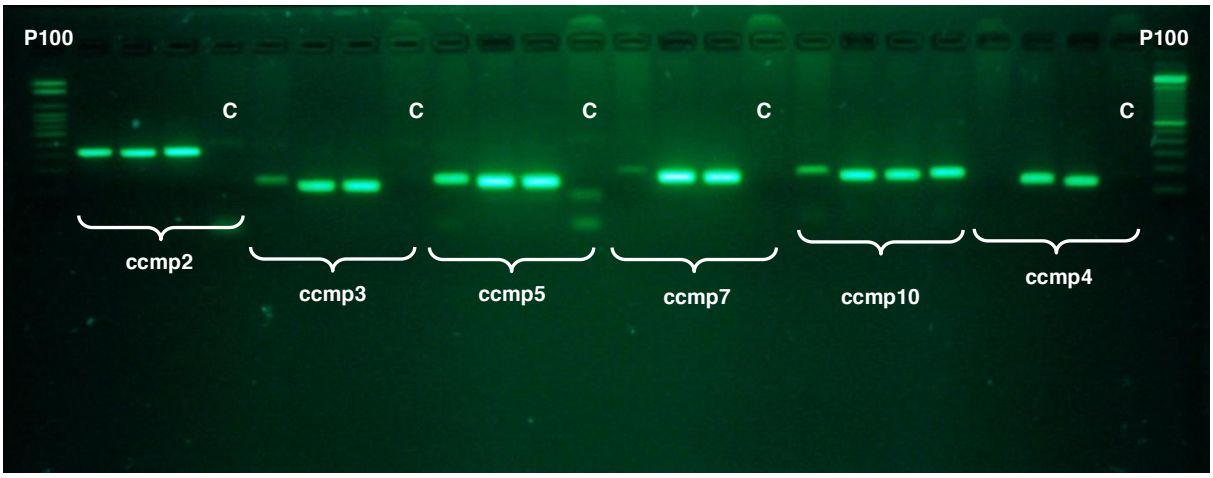


Figura 3: Produtos de PCR dos locos ccmp2, ccmp3, ccmp5, ccmp7, ccmp10 e ccmp4 visualizados em gel de agarose 2% amplificados nos indivíduos P3, P16 e P15 de *Croton floribundus* Spreng. P100 corresponde ao padrão de peso molecular de 100pb. C indica o controle da reação de PCR amplificada sem DNA.

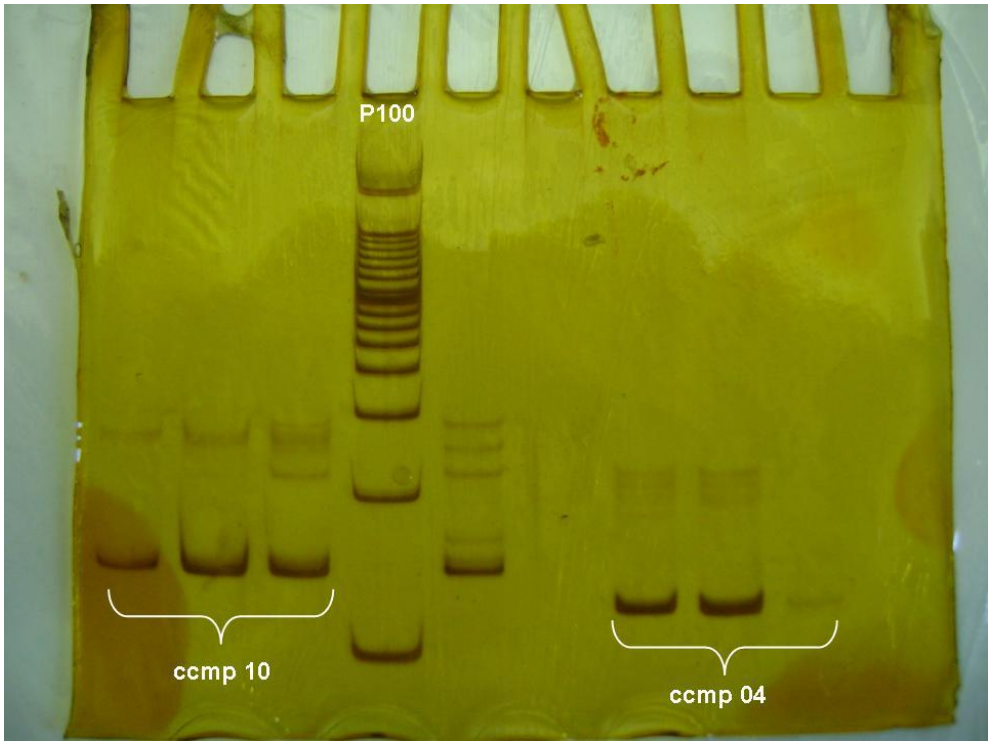


Figura 4: Produtos de PCR dos locos ccmp10 e ccmp4 visualizados em gel de poliacrilamida 6% não desnaturante amplificados nos indivíduos P3, P16 e P15 de *Croton floribundus* Spreng. P100 corresponde ao padrão de peso molecular de 100pb.

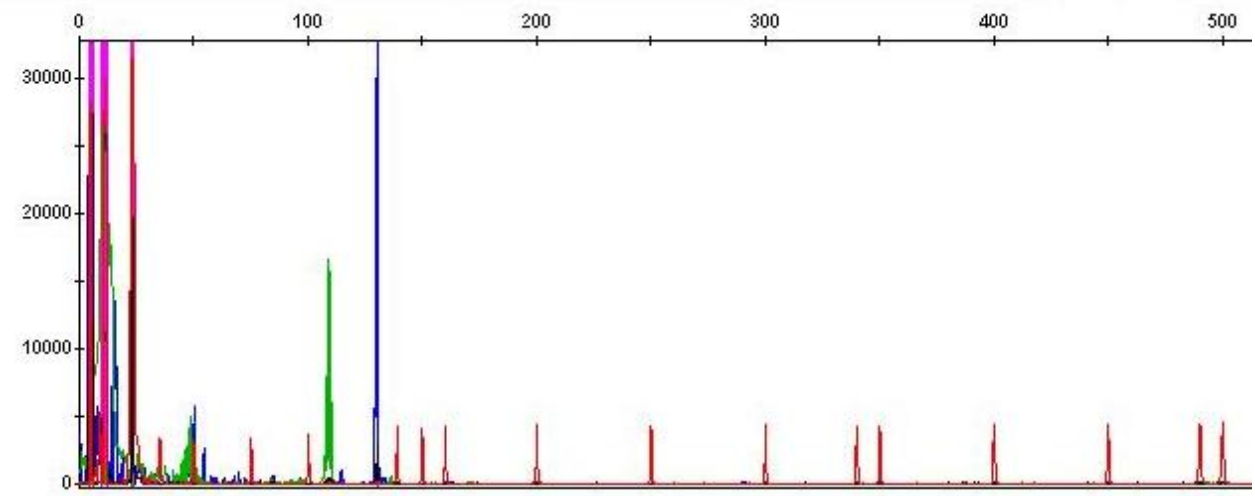


Figura 5: Eletroferograma do loco ccmp5 marcado com 6-HEX (verde) e do loco ccmp7 marcado com 6-FAM (azul) em *multiplex* amplificados no indivíduo P48 de *Croton floribundus* Spreng. O eixo x representa o tamanho dos fragmentos em pares de bases (pb) estimados por comparação ao padrão de peso molecular (vermelho). O eixo y representa as unidades relativas de fluorescência.

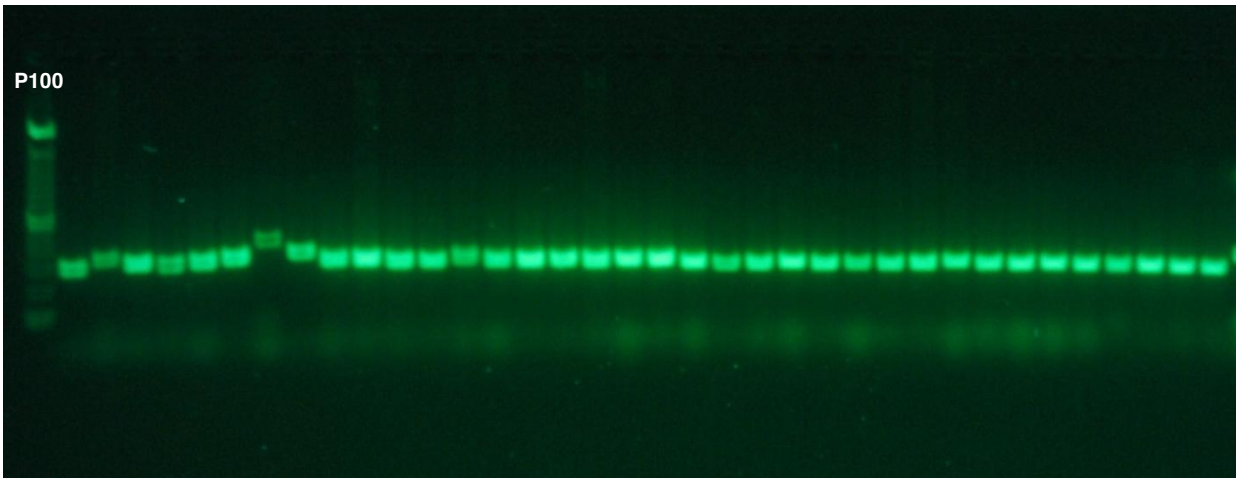


Figura 6: Produtos de PCR do loco ccmp2 marcado com 6-FAM (azul) visualizados em gel de agarose 2%. Da esquerda para direita os indivíduos de *Croton floribundus* Spreng.: P48, P50, P52, P54, P55, P56, P57, P58, P60, P61, P63, P78, P79, P80, P81, P82, P83, P84, P30, P31, P32, P33, P34, P36, P37, P38, P39, P40, P41, P42, P43, P45, P46, P47, P64 e P65. P100 corresponde ao padrão de peso molecular de 100pb.

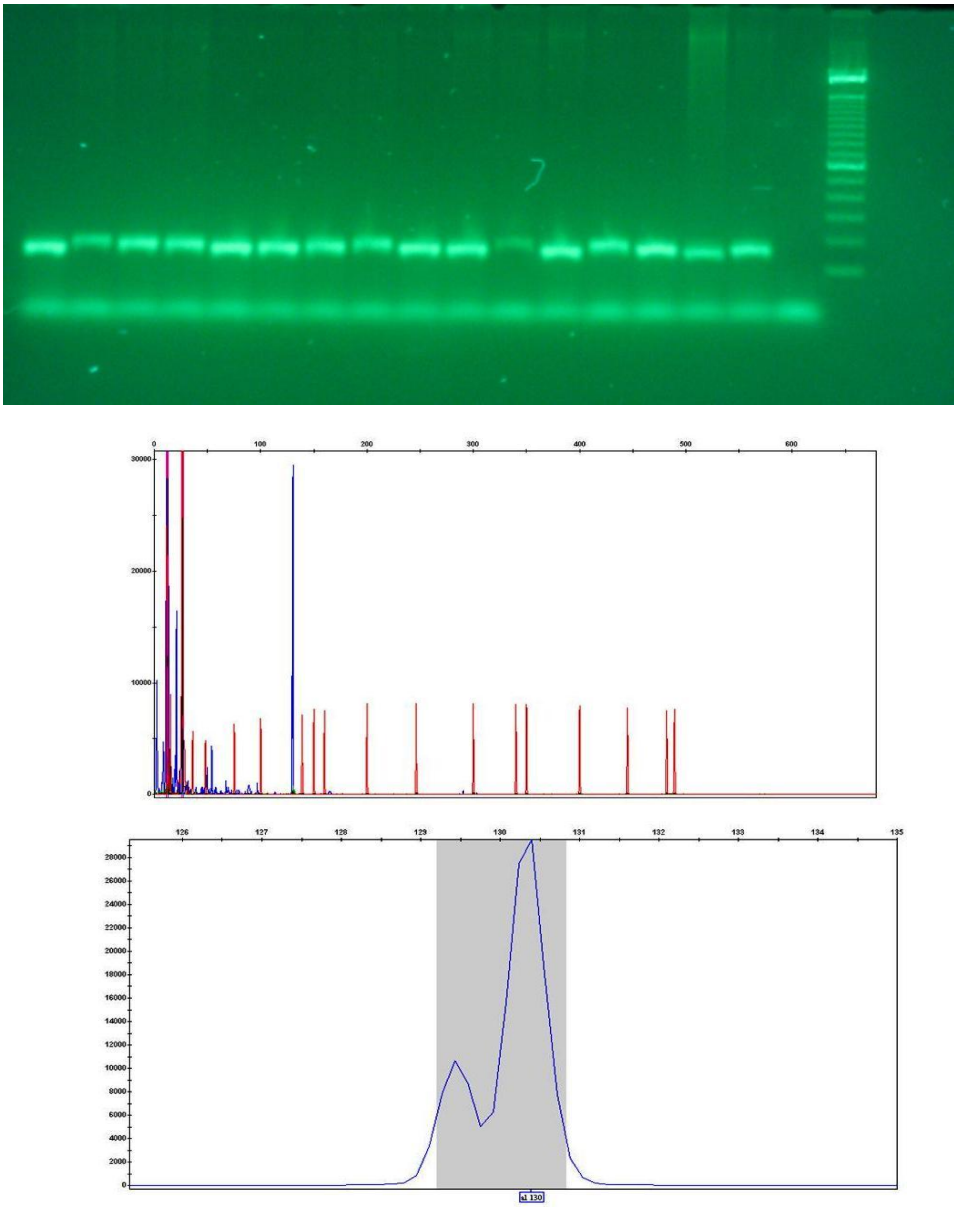


Figura 7: Produtos de PCR do loco ccmp7 marcado com 6-FAM (azul) visualizados em gel de agarose 2%. Da esquerda para direita os indivíduos de *Croton floribundus* Spreng: P9, P11, P12, P14, P15, P16, P17, P18, P19, P24, P25, P26, P28, P29, P27 e P8. P100 corresponde ao padrão de peso molecular de 100pb (a). Eletroferograma do loco ccmp7 marcado com 6-FAM (azul) mostrando o pico do alelo de 130pb amplificado no indivíduo P25 e os picos do padrão de peso molecular GENESCAN-500 ROX (Applied Biosystems, Inc.) (em vermelho) (b). Ampliação do eletroferograma do loco ccmp7 marcado com 6-FAM (azul) mostrando o pico do alelo de 130pb do indivíduo P25 (c). O eixo x representa o tamanho dos fragmentos em pares de bases (pb) estimados por comparação ao padrão de peso molecular. O eixo y representa as unidades relativas de fluorescência.

CHAPTER III

Cytogenetics and characterization of microsatellite loci for a South American pioneer tree species, *Croton floribundus* Spreng. (Euphorbiaceae).

Cytogenetics and characterization of microsatellite loci for a South American pioneer tree species, *Croton floribundus*.

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Abstract

Despite the recent advances in plant population genetic studies, the lack of information regarding pedigree, ploidy level or mode of inheritance for many polyploids can compromise the analysis of the molecular data produced. The aim of this study was to examine both microsatellite and cytogenetic characteristics of the pioneer tree *Croton floribundus* Spreng. (Euphorbiaceae) in order to test for the occurrence of polyploidy in the species and to evaluate its implications for the appropriate use of the SSR markers. Seven microsatellite markers were developed and screened for 62 individuals from a semi-deciduous tropical forest in Brazil. Chromosome number, meiotic behavior, and pollen viability were evaluated from male flower buds. All SSR loci were highly polymorphic. The number of bivalents observed in meiosis n=56 (2n=8x=112) and the maximum number of alleles per individual (N_i=8) demonstrated the occurrence of polyploidy in *C. floribundus*. The normal meiotic pairing and the high pollen viability suggested that *C. floribundus* is a regular and stable polyploid, most likely an allopolyploid. The combined SSR and cytogenetic data provided new evidence on the origin and evolution of the species as well as assured the accurate use of SSR loci for population genetic studies of the polyploid pioneer species.

Key words: chromosome number; meiosis; polyploidy; primers; semi-deciduous tropical forests; simple sequence repeat.

Introduction

Pioneer tree species exhibit particular life histories and population structures that are mainly affected by the patchy dynamics of the tropical forests (Swaine and Whitmore 1988; Alvarez-Buylla 1994; Uriarte et al. 2012). These characteristics and ecological processes have significant consequences for genetic structure and evolution (Alvarez-Buylla and Garay 1994; Alvarez-Buylla et al. 1996; Jones and Hubbell 2006), however, this is still poorly understood. Most genetic information available considers only a few species in a range of gap-phase regeneration behavior of the pioneer species (Brokaw 1987), mainly those that occur only in large gaps (Alvarez-Buylla et al. 1996; Born et al. 2008). Knowledge of the genetic characteristics of the pioneer species may help understand the effects of human disturbances and the succession processes in the tropical forests and, therefore, forest maintenance and restoration.

Croton floribundus Spreng. (Euphorbiaceae) is a shade-intolerant, fast-growing pioneer tree species commonly found in gaps and in secondary areas of the semi-deciduous tropical forests of the countryside of São Paulo, as well as other forests of Brazil and Eastern Paraguay (Gandolfi et al. 1995; Rodrigues 2005; Gomes 2006). According to the pattern of distribution of the species in gaps (M. Silvestrini and F.A.M. Santos, unpublished data, 2013), *C. floribundus* may be included into the subgroup of pioneer species that are widespread and common along the whole gap-size gradient and colonize over extended time. This gap-phase regeneration behavior is important because it allows the study of a more heterogeneous environment and its effects on genetic diversity and structure of the pioneer species. In other words, *C. floribundus* is part of a still genetically unknown group of species that has in turn an important role in the ecological and evolutionary processes of tropical forests.

Microsatellites or simple sequence repeats (SSR) are tandemly repeated sequences of one to six base pairs found in the genomes of almost every known organism and organelle (Chambers and MacAvoy 2000; Ellegren 2004). The markers exhibit multiallelic nature, neutrality, codominant inheritance, relative abundance, extensive genome coverage and simple detection by polymerase chain reaction (PCR) using two unique primers that flank the microsatellite and hence define the microsatellite locus (Powell et al. 1996; Zane et al. 2002). As a result, microsatellites are one of the most powerful and popular markers used to characterize genetic variability within and among populations (Guichoux et al. 2011).

Nonetheless, the accurate and efficient genotype identification of SSR loci can be compromised for polyploid organisms because of the difficulty or impossibility of allelic copy number determination and genotype assignments (Kosman and Leonard 2005; Obbard et al. 2006; Clark and Jasieniuk 2011). There are alternative methods such as those that allow allele copy number ambiguity assuming polysomic inheritance or code each allele as present or absent as multilocus fingerprints with loss of genetic information (see a brief review in Clark and Jasieniuk 2011). In any instance, the appropriate SSR scoring and analysis require knowledge of the ploidy level and the mode of inheritance of the species.

Here, we developed and characterized SSR loci for *C. floribundus*. Our preliminary SSR analyses showed more than two SSR alleles per individual, which strongly supported polyploidy and lead us to investigate the cytogenetic traits of the species. Polyploidy is very common in the family Euphorbiaceae (48% of the known taxa, Hans 1973), however, 74% of cytogenetic records of the genus *Croton* L. showed chromosome number of 2n=20, including species of same section of *C. floribundus* (Lasiogyne or Argyroglossum), such as *Croton blanchetianus* Baill., as well as species of the closely related section Julocroton (Berry 2001; Berry et al. 2005; Pôrto

2007; van Ee et al. 2011). Thus, despite the high likelihood of finding ploidy level similar to its sister species, we expect to find a higher chromosome number in *C. floribundus*.

Hence, in order to properly develop and characterize the SSR markers for *C. floribundus*, we also evaluated chromosome number, meiotic behavior, and pollen viability with the aim of investigating the occurrence of polyploidy in the species and evaluating its implications for the analysis and use of the SSR markers.

Materials and methods

Plant material

Genomic DNA of *C. floribundus* was used for construction of all genomic libraries. Microsatellite loci were characterized for 62 individuals of the life stage juveniles 1 (Danciguer 1996), hereafter referred to as juveniles. Juveniles are individuals of 5-37 cm height and 0.05-0.40 cm diameter at soil height. The juveniles were sampled at four gaps found within a 10.24 ha permanent plot in the primary forest at Caetetus Ecological Station (22°20' - 22°30'S; 49°40' - 49°45'W), Gália/Alvinlândia, southeast Brazil. We chose to sample this size class for the following two reasons: i) individuals were more easily assigned to a specific gap than a tree and this is important for future population studies that consider species distributions in the gaps; ii) the genetic diversity levels for other pioneer species have not differed between trees and samplings or seedlings (Alvarez-Buylla and Garay 1994; Jones and Hubbell 2006; Born et al. 2008). Plant material of two reproductive individuals from this population was deposited at UEC Herbarium, University of Campinas, Campinas, Brazil (accession numbers 172052 and 172054). Total genomic DNA was extracted from young leaves according to Doyle and Doyle (1990), using CTAB as detergent. DNA quality and quantity was checked on agarose (0.8% w/v) gels

stained with SYBR Safe® (Invitrogen Corporation, Carlsbad, CA, USA). DNA quantity was estimated by comparing the intensity of fluorescence emission of each sample with that of the lambda (λ) DNA standards (25, 50, 100, 200 and 300 ng). All DNA samples were diluted to a final concentration of 20 ng/μL.

Cytogenetic analyses were carried out on pollen mother cells (PMC) and pollen grains of male flower buds. Inflorescences were collected from four individuals of *C. floribundus* at Santa Genebra Reserve, Campinas (22º48' - 22°50'S, 47º06' - 47°07'W); two individuals in the forest remnant of Ribeirão Cachoeira, Sousas, Campinas (22°50'13"S, 46°55'58"W); and four individuals in forests at the region of Caetetus Ecological Station, Gália/Alvinlândia (22º20' - 22º30'S; 49º40' - 49º45'W), Brazil. In total, three individuals were completely analyzed (one individual per area). This is because we did not find suitable buds with PMCs in the development stage for chromosome counting in all sampled individuals. Plant material from two individuals was deposited at UEC Herbarium (accession numbers 172052 and 172053). Plant material from individuals from Santa Genebra Reserve is already well represented at UEC Herbarium (for instance, see accession numbers 58744 and 32541). For pollen viability analyses, only three individuals were evaluated, all of them sampled at Santa Genebra Reserve.

Construction of SSR-enriched libraries, SSR isolation and sequencing

Genomic DNA was enriched for repeat motifs following the methods of Billotte et al. (1999) with modifications. DNA from a randomly chosen individual of *C. floribundus* was digested by the enzyme *Rsa*I (New England Biolabs, Inc., Ipswich, MA, USA) and the fragments were ligated to the adaptors generated by annealing *Rsa*21 (5′-CTCTTGCTTACGCGTGGACTA - 3′) and *Rsa*25 (5′-

TAGTCCACGCGTAAGCAAGAGCACA - 3′). The adaptor-ligated genomic fragments were PCR-amplified and selected by biotin-labeled streptavidin-associated magnetic beads with the probes $(TTC)_8$, $(CT)_8$ and $(GT)_8$. The selected fragments were amplified by PCR using the primer *Rsa*21 (5′- CTCTTGCTTACGCGTGGACTA - 3′) and cloned into pGEM-T vectors (Promega Corporation, Fitchburg, WI, USA) that were subsequently transformed into competent XL1-Blue cells. Sequencing reactions were carried out using a SP6 primer and Big Dye 3.1 (Applied Biosystems, Inc., Foster City, CA, USA) in a MyCycler Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Sequences were run on an automated sequencer 3730 DNA Analyzer (Applied Biosystems).

We sequenced 96 inserts, of which sequences containing microsatellites were aligned and assembled using Clustal X 1.83 (Thompson et al. 1999) and Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA).

The identification of SSR in this study was based on the assumption that 10 nucleotides in tandem are necessary to initiate the origin of a SSR (Schlötterer 1998). Hence, the following parameters were used: for mononucleotides, a minimum number of 10 repeats, for dinucleotides a minimum number of 5 repeats, for tri 4 repeats and for tetra, penta, and hexanucleotides, 3 repeats. Microsatellite sequences were classified into the following four main categories: perfect, compound, complex and interrupted repeat sequences (Chambers and MacAvoy 2000). We followed the alphabetical rules of Chambers and MacAvoy (2000) for representation and naming of microsatellites. Besides the number and quality of SSR sequences, the efficiency of library enrichment was evaluated by calculating the level of redundancy, which is the number of clones with redundant sequences divided by the total number of sequences that contained SSR.

Primer design

Sixteen primer pairs (Table 1) were designed, synthesized, tested, and optimized for SSR loci amplification in *C. floribundus*. For the primer design, the PRIMER3 software (Koressaar and Remm 2007; Untergasser et al. 2012) was used with primers 20 nucleotides long, an annealing temperature ranging from 57 to 62°C and GC content around 50% as the main selection criteria. Primer quality was checked using GeneRunner v. 3.1 software (Hastings ***Software,*** Inc., Hastings, NY, USA) and sequencing quality in the region of primer was verified with Chromas 2 software (Technelysium Pty Ltd., Helensvale, Australia).

PCR amplification and polymorphism visualization

The optimization process was carried out using increasing PCR stringency conditions, which consisted of raising temperature and reducing concentration of Mg++ ions. The primer pairs were tested for gradients of temperature between 50°C and 60°C, 55°C and 64°C, and 60°C and 70°C; and for $MgCl_2$ concentration of 2.0, 1.5 and 1.0 mmol/L when it was necessary. Concentrations of DNA of 20, 40, and 80 ng and dNTP of 0.1 and 0.2 mmol/L were tested for primers SSR-2, SSR-9, and SSR-10.

After primer optimization, primer pairs were selected for fluorescent labeling. The forward primers were labeled with two different fluorophores according to the expected product size of each locus, 6-FAM for the longest and 6-HEX for the shortest product size. This procedure was carried out in order to perform multiplex genotyping, i.e., the differentiation of two SSRs in a single run. Yet we opted for not using multiplex genotyping in the locus characterization in order to facilitate clear scoring because of the high number of alleles amplified per individual. In order to improve the peak detection on the automated sequencer analyses, we tested the combination of the primer quantity in the reaction (10, 5, and 2.5 ρmol)

with the volume of reaction to be injected into the genetic analyzer capillary (0.5, 1.0, and 1.5 uL).

Final PCR conditions were set as follows in a volume of 25 µl: 40 ng of genomic DNA, 1x reaction buffer, 0.1 mmol/L of dNTP, 2 mmol/L of $MgCl_2$, 10 ρmol of each primer, and 1.0 U of *Taq* DNA polymerase. For primer SSR-6, we used 5 ρmol of each primer in an effort to minimize offscale peaks, and the optimal concentration of $MgCl_2$ was 1.0 mmol/L. The complete thermal cycle program was 5 min at 95°C; followed by 30 cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 72°C; and a final 5 min of elongation time at 72°C.

Amplification products, obtained using 5' end fluorescent dye labeled forward primers (6-FAM and 6-HEX), were analyzed on an automated sequencer 3730 DNA Analyzer (Applied Biosystems). All PCR reactions were previously analyzed by agarose gel electrophoresis to confirm SSR amplification. Genotypes were scored using Peak Scanner software v. 1.0 (Applied Biosystems). The size standard GENESCAN-500 ROX (Applied Biosystems) was co-injected into the genetic analyzer capillary with the sample to allow for the accurate determination of fragment sizes. The samples were prepared for sequencing using 1.5 µL PCR products, 9 µL formamide, and 0.5 µL size standard.

Characterization of SSR loci

SSR loci were scored by presence or absence of bands or alleles. As we assumed polyploidy in *C. floribundus*, we did not distinguish genotypes and estimate allelic frequencies as for co-dominant markers in diploids. Allelic frequencies (p_i) were estimated as the relative frequency of the j^{th} allele or band for each SSR marker in the population. For estimating gene

diversity (*H'*) and Shannon's genetic diversity index ($H'_{Shannon}$), however, the SSR markers were treated as multilocus fingerprints, in which each allele was either scored present or absent (p_i').

Patterns of allelic diversity at each locus were examined by estimating the following genetic parameters in the population (62 individuals distributed in four gaps): number of alleles per locus (N_a), number of alleles per individuals in each locus (N_i), effective number of alleles per locus (A_e), gene diversity (*H* and *H'*), and Shannon's genetic index ($H'_{Shannon}$).

N_a and N_i were determined by summing all observed alleles at each locus and each individual, respectively. A_e was calculated for each locus as $A_e = 1/\sum(p_i)^2$, where p_i is the frequency of the i^{th} allele or band. Gene diversity was estimated as the probability that two randomly chosen alleles or bands are different in the sample (*H*) and the probability that a specific allele or band is present and absent when two gene copies are sampled at random in the population (*H'*) (Nei 1987). For both approaches, we used the unbiased estimate of Nei (1987):

$$H = \frac{n}{n-1}\left(1 - \sum_{i=1}^{k} p_i^2\right)$$

where *n* is the number of individuals in the sample, *k* is the number of alleles or bands, and p_i is the sample frequency of the i^{th} allele. For *H'*, we substituted p_i by p_i', which is the frequency of the presence or absence of the allele or band in the population.

$H'_{Shannon}$ for each allele or band was calculated according to Bussell (1999) as:

$$H'_{Shannon} = -\sum p_i' \log_2 p_i'$$

As suggested by Bussell (1999), monomorphic bands or alleles were included in all analyses in order to allow invariant loci to influence diversity values.

Cytogenetic analyses

Chromosome number was counted from pollen mother cells (PMC) of male flower buds. Flower buds were fixed in Carnoy solution (3 parts alcohol: 1 part glacial acetic acid), and stored in the freezer. The cytological preparations were obtained by squashing anthers in 1.2% aceto-carmine (anthers of one flower bud per slide). Seven, eight, and 10 slides per plant were observed in individuals sampled at Ribeirão Cachoeira, at the region of Caetetus Ecological Station, and at Santa Genebra Reserve, respectively. In an attempt to quantify anomalous meiotic behavior, the number of cells that showed at least one irregularity was computed as an abnormal cell. In the same way, tetrads with normal or abnormal number of microspores were recorded. Chromosome number determination was based on the observation of two and five PMCs per individual (two individuals). For pollen viability analyses, fresh anthers of one floral bud per plant (three individuals) were crushed between the slide and the coverslip in a drop of Alexander stain - basic stain with 4ml of lactic acid (Alexander 1980) and observed under light microscopy directly. Alexander stain with six different concentrations of lactic acid was previously tested in order to obtain the appropriate coloration according to the thickness of the pollen grain wall. In the fertile or viable pollen grains, the Alexander test revealed the cytoplasm and nuclei were stained, while in sterile or inviable pollen the grains were empty. The number of viable and sterile pollen grains was counted in one slide per individual on a coverslip area of 24 mm x 24 mm. The percentage of viable pollen was calculated by dividing the number of viable pollen per total number of pollen grains analyzed. Since data on proportions and percentages follow a binomial distribution (Zar 2010), arcsine transformation of data (square root of p) was used in order to make the distribution normal and thus appropriately obtain mean and standard error estimates. Complementary pollen analyses were carried out on fixed pollen grains that were stained with 1.2% aceto-carmine for individuals sampled at Ribeirão Cachoeira and the region of Caetetus Ecological Station.

Results

Microsatellite-enriched libraries

Seventy-one of the 96 inserts (73.96%) sequenced contained at least a single microsatellite region. The level of redundancy was 19.7%, which means that 14 clones harbored redundant repeats. From the 57 clones with unique SSRs, a total of 87 SSR regions were found, of which 35.6% were dinucleotides, 29.9% tri, 20.7% mono, 6.9% tetra and 1.1% pentanucleotides. Also, 5.8% were a compound of two different repeats. Excluding the mononucleotides, we obtained the following distribution of repeats: 44.9% di, 37.7% tri, 8.7% tetra, 1.5% pentanucleotides and 7.2% were a compound of two different repeats. Given the quality of sequences and position of SSR repeats at the sequences at least 32 SSR primer pairs could be designed to amplify microsatellite loci.

In total, 78 unique SSR loci (89.7%) had perfect structure, three (3.4%) had compound structure, four (4.6%) had interrupted compound structure, and two (2.3%) were interrupted complex. All 18 mononucleotide motifs had perfect structure. Apart from them, the majority of perfect SSRs were dinucleotide and trinucleotide in the proportion 45% and 43.3%, respectively. The most frequent perfect motifs were the repeated units used in enrichment $(GT)_n$ (30.0%), $(CT)_n$ (15.0%), $(TTC)_n$ (38.3%), followed by $(AGG)_n$ (3.3%), $(ATA)_n$ (1.7%), $(CCTCT)_n$ (1.7%), and the remaining (10.0%) were of five distinct types of tetranucleotides (mononucleotides excluded).

Primer screening

Of the total of 16 primer pairs designed and tested, five were unable to amplify products under all PCR conditions tested. The remaining primers that produced the expected size product

without nonspecific amplifications after optimization were fluorescent labeled (6-FAM and 6-HEX) in order to be genetically characterized. However, loci SSR-1, SSR-2, SSR-9, and SSR-11 did not produce unambiguously scorable markers as expected after preliminary analyses. They presented PCR artefacts such as stutter bands and triallelic patterns and were discarded.

SSR characterization

Seven SSR loci were fully characterized in order to be used in population genetic studies (Table 2). All analyzed loci were polymorphic. A total of 69 alleles were obtained from the seven loci. The average number of alleles per locus was 9.9. All loci, except for SSR-8, presented rare alleles, i.e., alleles that occur in only one or two individuals. SSR loci amplified more than two alleles per individual in *C. floribundu*s, with the exception of loci SSR-8 and SSR-14 (Table 2). The maximum number of amplified alleles (N_i) per individual was eight observed in loci SSR-10 and SSR-16 (Table 2).

Locus SSR-12 presented the greatest number of alleles (N_a) followed by SSR-10 and SSR-16 (Table 2). Loci SSR-5, SSR-10, SSR-12, SSR-14, and SSR-16 presented variation in size different than that expected by the repeat unit length. Loci SSR-5 and SSR-12 presented periodicity of one, two, and three base pairs instead of only three as would be expected from their $(AAG)_8$ and $(GAA)_8$ repeat motifs. Based on this result, we went back to the original sequence where the primers were designed and found a dinucleotide motif located in the region of amplified sequence that has not been considered as a microsatellite earlier (<10 nucleotides in tandem). Note that this was the reason for adding the additional dinucleotide motifs in each locus on Tables 1 and 2.

For the other loci, however, we did not identify a different SSR region on the sequence of amplified locus. In this case, we just found what we call sets of alleles, i.e., groups of alleles that

differed in the size periodicity between each other. For instance, SSR-14 showed the sets 192-198 bp and 193-195 bp. Both sets showed a variation of two base pairs from one allele to other but if we consider all alleles, we had differences in one and three base pairs. SSR-10 showed the following sets of alleles: 141-147 bp, 151-178 bp, and apart from them the allele 187 bp. The two first sets presented a variation between alleles of three base pairs. SSR-16 showed the sets 200 to 203 bp, 208 to 226 bp, and 243 to 255 bp. The alleles of different sets were amplified concomitantly in the same individual, except for SSR-14.

Besides the higher variation from locus to locus, gene diversity estimated using p_i (H) showed much higher values than when it was calculated based on p_i' (H' and $H'_{Shannon}$) (Table 2). The differences among coefficients were more prominent for those loci which had a higher number of alleles per locus (N_a) and amplified a higher number of alleles per individual (N_i) (Table 2). The main reason is that H considers both inter- and intra-individual variation, the latter being higher for individuals with higher N_i, while H' and $H'_{Shannon}$ evaluate only inter-individual genetic diversity.

Cytogenetic analyses

Chromosome number counted from pollen mother cells of the individuals sampled at Caetetus Ecological Station (five cells) and Santa Genebra Reserve (two cells) varied from n=53 to n=58, but in the best chromosome spreads and quality of images (one cell from each population), we found 56 bivalents (n = 56) (Fig. 1).

In the individual sampled at Ribeirão Cachoeira, Sousas, Campinas, a total of 33 cells were analyzed but we could not accurately estimate the chromosome number. Cytoplasm of cells presented too much viscosity and we did not find enough spread of chromosomes for counting.

By the volume of bivalents aligned at the metaphase plate, however, we could infer a similar chromosome number to the previously analyzed populations (Figs. 2*a*, 2*c*).

In the majority of cells analyzed from individuals sampled at Caetetus Ecological Station and Santa Genebra Reserve, bivalents showed normal pairing at metaphase I, anaphase I, and telophase I and II (Figs. 1, 2*a*, 2*b*), but 34.8% had some irregularity. Homogeneous tetrads were observed as well (Figs. 3*a*, 3*e*), but 4% were irregular. In the individual sampled at Ribeirão Cachoeira, 84.8% of cells presented abnormal diakinesis, metaphase I and II, anaphase I and telophase I (Figs. 2*c*, 2*d*). Also, instead of only normal tetrads of microspores, 75.9% of polyads were formed (Fig. 3*c*). Note that a cell was computed as having abnormal pairing when at least one irregularity was detected, for example occurrence of one multivalent, early segregation or one chromosome lagging (Figs. 2*c*, 2*d*).

The mean pollen viability evaluated in *C. floribundus* anthers was 87.12 ± 0.03% (Santa Genebra Reserve) (Fig. 3*f*). The normal tetrads of microspores (Fig. 3*e*) and the pollen grains with homogeneous size (Fig. 3*f*) confirmed regularity in the meiotic behavior (Fig. 1*b*). By contrast, for individuals sampled at Caetetus Ecological Station (Figs. 3*a*, 3*b*) and Ribeirão Cachoeira (Figs. 3*c*, 3*d*), pollen grains varied in size and did not follow the predominant pattern of microspore formation. In the first individual that had 96% of regular tetrads (Fig. 3*a*), we recorded 50% of slides with balanced size of pollen grains (Fig. 3*b*); while in the second, that presented 75.9% of abnormal tetrads (Fig. 3*c*), the size of pollen grains was homogeneous in 80% of slides (Fig. 3*d*). The great discrepancy between abnormality of meiosis and size regularity of the pollen grains for the individual sampled at Ribeirão Cachoeira is likely a result of a skewed sampling of damaged anthers during meiosis analyses since we observed differences in size, rugosity, and color in most of the analyzed anthers in this individual.

Discussion

Of the 16 tested primers, seven were useful for population genetic studies. Five loci did not amplify and another four loci presented excessive stutter bands and triallelic patterns under all PCR conditions tested. According to Guichoux et al. (2011), triallelic patterns are caused by the mispriming of primers and when the artefacts interfere with allele calling the primers should be excluded. For these loci, we could not distinguish real alleles from artefacts mainly because of production of several alleles per individual in the polyploid and similar variation in size (usually two base pairs) for both real alleles and stutter or triallelic pattern peaks.

The four excluded loci, SSR-1, SSR-2, SSR-9, and SSR-11, presented more than 17 repeated units and, apart from SSR-1, had dinucleotide repeats. In contrast, of the seven characterized loci, only SSR-10 and SSR-16 showed slight stutter bands that did not affect SSR scoring, and all of them presented a low number of repeat units (5 to 11 repeat units). Our results confirmed that dinucleotide and longer repeat arrays are more prone to stutter bands (Chambers and MacAvoy 2000; Ellegren 2004). Primers designed from SSRs with short repeat unit length and high number of repeat units were less successfully amplified compared with other SSR types in the North American tree *Cornus florida* L. (Wang et al. 2008). Although those SSR characteristics may assure the highest level of polymorphism for some species (Schlötterer 1998), they were a problem for scoring *C. floribundus*, perhaps owing to the high ploidy level and genetic diversity of the species. This finding may be helpful for future development of SSR loci for tropical trees since polyploidy and high genetic diversity levels have been commonly found in these species (Hamrick et al. 1992; Caddah et al. 2009; Brito et al. 2010; Cavallari et al. 2010).

We used 10 nucleotides in tandem, criterion used by most investigators, to find SSR regions and design primers (Schlötterer 1998; Chambers and MacAvoy 2000). Nevertheless, we

had evidence that SSR regions of eight and six nucleotides (four and three dinucleotide repeats) changed in length by the observed variation in allelic size of loci SSR-5 and SSR-12. On the other hand, we have found other loci that also presented unexpected allelic periodicity without any sign of additional SSR motifs in the sequence. Besides the variable periodicity, the sets of alleles showed specific size range with considerable differences in length between each other. This type of variation may be a consequence of allelic amplification on divergent genomes of the polyploid. In this case, each set of alleles would be the result of point mutations and (or) insertions-deletions occurred in specific genomes before or more likely after formation of the polyploid. Indeed, according to Parisod et al. (2010), large structural genomic changes including insertion, deletion, and homogenization of repetitive sequences are frequent during process of polyploidization, especially in allopolyploids. Whatever the exact explanation is for the results of SSR-5 and SSR-12, we recommend the use of a more conservative approach for definition of SSR regions when developing microsatellites, particularly for species thought or expected to have high genetic diversity levels.

The seven developed SSR loci showed to be efficient for detecting polymorphisms in *C. floribundus*. The number of alleles per locus (N_a) was in the same range of other tropical trees (Ciampi et al. 2008; Guidugli et al. 2009), including polyploids (Brito et al. 2010; Cavallari et al. 2010) and even higher if we consider the sample sizes (Collevatti et al. 1999; Lowe et al. 2002). *H* values were in the same range of the expected heterozygosity found for *Caryocar brasiliense* Cambess. and *Cariniana estrellensis* (Raddi) Kuntze (Collevatti et al. 1999; Guidugli et al. 2009). The values were also compared to PIC values found for the polyploid tree *Tibouchina pulchra* (Cham.) Cogn., although the equations used for estimating both gene diversity indices were slightly different (Brito et al. 2010).

Metaphase I spreads of pollen mother cells showed 56 bivalents (n = 56). Despite the low number of individuals per population analyzed and the rough estimate for the individual sampled at Ribeirão Cachoeira, polyploidy was found in all populations. The n=56 was the second highest chromosome number recorded for the genus *Croton* (Berry 2001; Pôrto 2007). The first was n=60 for *Croton xalapensis* Kunth (section Cyclostigma). Another high chromosome number of n=32 was registered for species of the sections Cyclostigma and Lamprocroton (Berry 2001; Pôrto 2007) and n=24, 20 for species of section Velamea. All these species are considered polyploids based on a basic number of x=8 and x=10 (Pôrto 2007).

Authors have not agreed regarding the basic number of genus *Croton*. Urbatsch et al. (1975) suggested the number x=10 as the basic number of genus, while x=7, 8, and 9 would be secondarily derived by aneuploid loss. Pôrto (2007) in turn pointed out that genus *Croton* may be polybasic as suggested earlier by Bernardello et al. (1990) or might have evolved by disploidy and polyploidy from the primary basic number x=8, with x=11, 10, 9, and 7 being secondarily derived basic numbers.

In *C. floribundus*, the number n=56 may have been derived by polyploidy from x=14 or x=7. If the basic number is x=14, the species is an octoploid, which agrees with the SSR results. Conversely, many studies have indicated x=7 as one of the basic numbers in the genus *Croton* (Bernardello et al. 1990; Pôrto 2007), although there is no record of this chromosome number in any of the species studied (Urbatsch et al. 1975; Berry 2001; Pôrto 2007). In this case, x=14 would be an old tetraploid number derived from auto or allopolyploidization of x=7 ancestors (Urbatsch et al. 1975; Pôrto 2007) and the ploidy of *C. floribundus* would be 2n=16x=112.

In general, analysis of PMCs in metaphase, anaphase and telophase showed a low frequency of abnormal pairing, which indicated a regular meiosis in *C. floribundus*. The low frequency of multivalents suggested the predominance of a disomic mode of inheritance, i.e.,

when pairing occurs essentially between homologous chromosomes (Ramsey and Schemske 2002). This result has important implications for population genetic and evolutionary studies because the mode of inheritance affects the segregation of genetic variation in the offsprings (Stift et al. 2008) and consequently the choice of suitable genetic analysis methods. For example, the disomic inheritance in tetraploids implies that standard population genetic tools developed for diploid organisms cannot be applied in the species even if we know the dosage of alleles (Obbard et al. 2006). The reason is that we cannot distinguish which alleles are associated with which of the duplicate loci and then accurately determine the genotypes. Although the recent increase in computational statistics methods that allow to analyze polyploids assuming polysomic inheritance, e.g. in Structure (Falush et al. 2007) and Spagedi softwares (Hardy and Vekemans 2002), and even allotetraploids with nonrandom segregation (see Clark and Jasieniuk 2011), there is still no software available for allelic discrimination of polyploid SSR data with ploidy higher than 4x and disomic or intermediate inheritance (Clark and Jasieniuk 2011). Thus, for *C. floribundus*, even with the possible loss of genetic information, it seems that the multilocus DNA fingerprint approach is currently the more appropriate method to assess population genetic statistics.

The balanced segregation of chromosomes in meiosis probably has as consequence a high level of fertility (Madlung et al. 2005; Soltis and Soltis 2009), as the presence of multivalents or chromosome lagging would result in abnormal and sterile pollen grains. This was confirmed by the often homogeneous size of pollen grains and the high pollen viability found in the *C. floribundus* anthers (87%), which were similar to previous values recorded by Passos (1995) (90%). The viable pollen rates are not as high as *Croton suberosus* Kunth (means of 98 ± 2 %; Narbona and Dirzo 2010) and *Croton priscus* Croizat (96%, Passos 1995), but may be very high when compared to allopolyploids with abnormal meiosis (Madlung et al. 2005).

The high chromosome number, relatively regular meiosis and high percentage of viable pollen grains suggested that *C. floribundus* arose after duplications of chromosomes in a hybrid species, i.e., it was originated by allopolyploidy (Soltis and Soltis 2009). On the other hand, structural genomic changes and diploidization of autopolyploid genomes is also possible in polyploids (Ramsey and Schemske 2002; Parisod et al. 2010). In addition, the less frequent but observed presence of multivalents, irregular tetrads, and unbalanced size of pollen grains indicated a minor degree of similarity between genomes. Given the high ploidy level, it seems more likely, however, that the species have been originated primarily by hybridization of closely related species followed by some genomic autoduplications rather than exclusively by autopolyploidy.

SSR analyses corroborated the octoploidy in *C. floribundus*. The maximum number of alleles per individual (N_i) was eight, the maximum number of alleles in a completely heterozygous octoploid. This result supported x=14 as the basic chromosome number in *C. floribundus*. Additionally, the different sets of alleles found for some loci (different size periodicity of SSR alleles in the same locus), as well as the high intra-individual genetic diversity seem to be a product of the high genomic divergence pointed out by cytogenetic analyses.

The results revealed that *C. floribundus* is a regular and stable polyploid, most likely an allopolyploid with 2n=8x=112 chromosomes. This is the first record of polyploidy in the section Argyloglossum, which may be very important for understanding the evolution of the genus *Croton*. Furthermore, the high genetic diversity values found using the multilocus DNA fingerprint approach showed that the SSR loci have high discriminant power to evaluate patterns of genetic diversity and structure in the polyploid pioneer species. Our findings may be very helpful for future studies of macro- and microevolutionary questions associated to *C. floribundus*.

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Tables

Table 1. Locus code, respective forward (F) and reverse (R) primer sequences, expected product size, and repeat motifs of microsatellite loci designed for *Croton floribundus* Spreng.

Locus code	Sequence of primers (5'- 3')	Product size	Repeat motif
SSR-1	F= CAGCATCAGAATCAATGTTGTC R= CCATCTCTGCATTTCACTCC	248	$(GAA)_{24}(GA)_3GTG(GAA)_2$
SSR-2	F= GCTTCTGCTGCTTCTGGTTTAT R= TAGCTCCCTGCAAAATCCAC	178	$(AG)_{17}$
SSR-3	F= GATGATGAGGAGGAGGAGGA R= GACATGGCTGCAAAGACAGA	172	$(AAG)_{19}$
SSR-4	F= GGAGGCTCTGCATTACAAGG R= GACAAAGGAGGAGGGTTGC	224	$(AGA)_8$
SSR-5	F= CGTGTGCTTCCTCTTCACC R= GCCAAATGGGTTTCTCGTTA	174	$(AAG)_8N_{18}(CT)_4$
SSR-6	F= CCACATTCCACATCACTTCC R= ACGTGGCAAAATGGGTAGTC	209	$(GA)_5$
SSR-7	F= TGGACTCAAGTTACAAGCCAAT R= AGGCTTTCTGGAATCCAACG	183	$(AGA)_7$
SSR-8	F= CTCCTGAACCCACGCTAGAT R= AGAGGCTTCGTGTTGTTCGT	216	$(AG)_8$
SSR-9	F= CGTTAGGGAATTGTGTCGATT R= ATCCTGTGGTCGCAAGAGAA	231	$(TC)_{21}TGATT(TC)_3$
SSR-10	F= AGACGGAAGGGAAGTGGAGT R= CGCAGCATCATATCACCAGA	160	$(AAG)_8$
SSR-11	F= CTAGCCAAAACCAAGCCAAA R= TGACTCCGTTGGAGAATCAGT	244	$(AC)_{15}(TC)_{10}$
SSR-12	F= TTGGGCAGATTCATGTAACG R= TTGGTTGAGGGAACAGATGA	172	$(GAA)_8N_{37}(AG)_3$
SSR-13	F= GATGGCATTCACAACAGCTT R= TTCGGGCTTACCTTAGTGGT	177	$(GAA)_9$
SSR-14	F= AGCACTCCTTCCACGACATT R= TGGTGAGACGCTTCATGTTG	212	$(CA)_{11}$
SSR-15	F= CTGTGCTTCGATGGGAGAG R= CGGGATTCTCTTGCTTACG	255	$(CT)_{12}(GT)_9$
SSR-16	F= GGCAGGCATAAATCAGCAAT R= TATCGAATCGTCGGATTTGG	223	$(GAA)_6$

Table 2. Characteristics of seven microsatellite loci amplified in 62 individuals of *Croton floribundus* Spreng. sampled at four gaps in the primary forest at Caetetus Ecological Station, Gália/Alvinlândia, Brazil.

Accession number[a]	Locus code	Sequence of primers (5'- 3')[b]	Repeat motif	Size range[c]	N_a[d]	N_i[e]	A_e[f]	H[g]	H'[h]	$H'_{Shannon}$[i]
JX236034	SSR-5	F= CGTGTGCTTCCTCTTCACC R= GCCAAATGGGTTTCTCGTTA	$(AAG)_8N_{18}(CT)_4$	142-183	13	3-7	6.5	0.860	0.210	0.478
JX236034	SSR-6	F= CCACATTCCACATCACTTCC R= ACGTGGCAAAATGGGTAGTC	$(GA)_5$	203-213	6	1-3	2.8	0.647	0.208	0.462
JX236035	SSR-8	F= CTCCTGAACCCACGCTAGAT R= AGAGGCTTCGTGTTGTTCGT	$(AG)_8$	214-216	2	1-2	2.0	0.497	0.195	0.412
JX236036	SSR-10	F= AGACGGAAGGGAAGTGGAGT R= CGCAGCATCATATCACCAGA	$(AAG)_8$	141-187	14	4-8	8.1	0.891	0.244	0.553
JX236037	SSR-12	F= TTGGGCAGATTCATGTAACG R= TTGGTTGAGGGAACAGATGA	$(GAA)_8N_{37}(AG)_3$	157-198	15	2-6	7.7	0.885	0.262	0.574
JX236038	SSR-14	F= AGCACTCCTTCCACGACATT R= TGGTGAGACGCTTCATGTTG	$(CA)_{11}$	192-198	5	1-2	2.6	0.624	0.251	0.555
JX236039	SSR-16	F= GGCAGGCATAAATCAGCAAT R= TATCGAATCGTCGGATTTGG	$(GAA)_6$	200-255	14	3-8	7.5	0.881	0.237	0.540

[a] Accession number of nuclear microsatellite loci in the "Genebank"
[b] Forward (F) and reverse (R) primer sequence
[c] Product size range in base pairs
[d] Number of alleles per locus
[e] Number of alleles per individuals in each locus
[f] Effective number of alleles per locus
[g] Gene diversity based on p_i
[h] Gene diversity based on p_i'
[i] Shannon's genetic index

Figure captions

Fig. 1. Bivalents in meiotic metaphase I cells of *Croton floribundus* Spreng. in individuals sampled at the region of Caetetus Ecological Station, Gália/Alvinlândia (*a*) and at Santa Genebra Reserve, Campinas (*b*). Pollen mother cells stained with 1.2% aceto-carmine stain.

Fig. 2. Meiosis in *Croton floribundus* Spreng. Pollen mother cells (PMC) at metaphase I and telophase I and II in the individual sampled at the region of Caetetus Ecological Station, Gália/Alvinlândia (*a*, *b*). Metaphase I with early segregation (arrows) and telophase I with laggards (arrowheads) in the individual sampled at Ribeirão Cachoeira, Sousas, Campinas (*c*, *d*). PMCs stained with 1.2% aceto-carmine stain.

Fig. 3. Meiotic products of *Croton floribundus* Spreng. in individuals sampled at the region of Caetetus Ecological Station, Gália/Alvinlândia (*a*, *b*); at Ribeirão Cachoeira, Sousas, Campinas (*c*, *d*); and at Santa Genebra Reserve, Campinas (*e*, *f*). Note the regular (*a*, *e*) and irregular tetrads (*c*), and the pollen grains with homogeneous (*d*, *f*) and heterogeneous size (*b*). Meiotic products stained with 1.2% aceto-carmine (*a*-*e*) and Alexander stain (*f*).

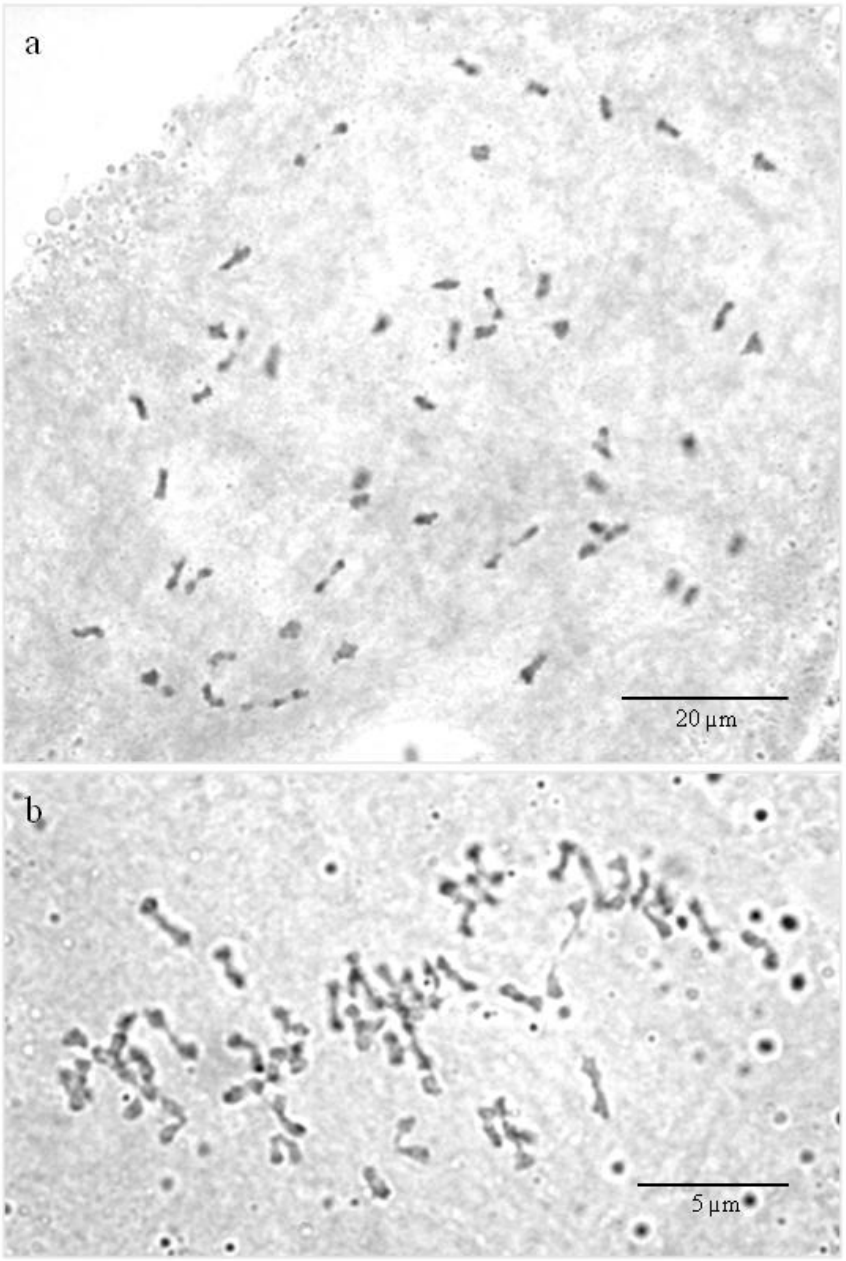

Fig. 1

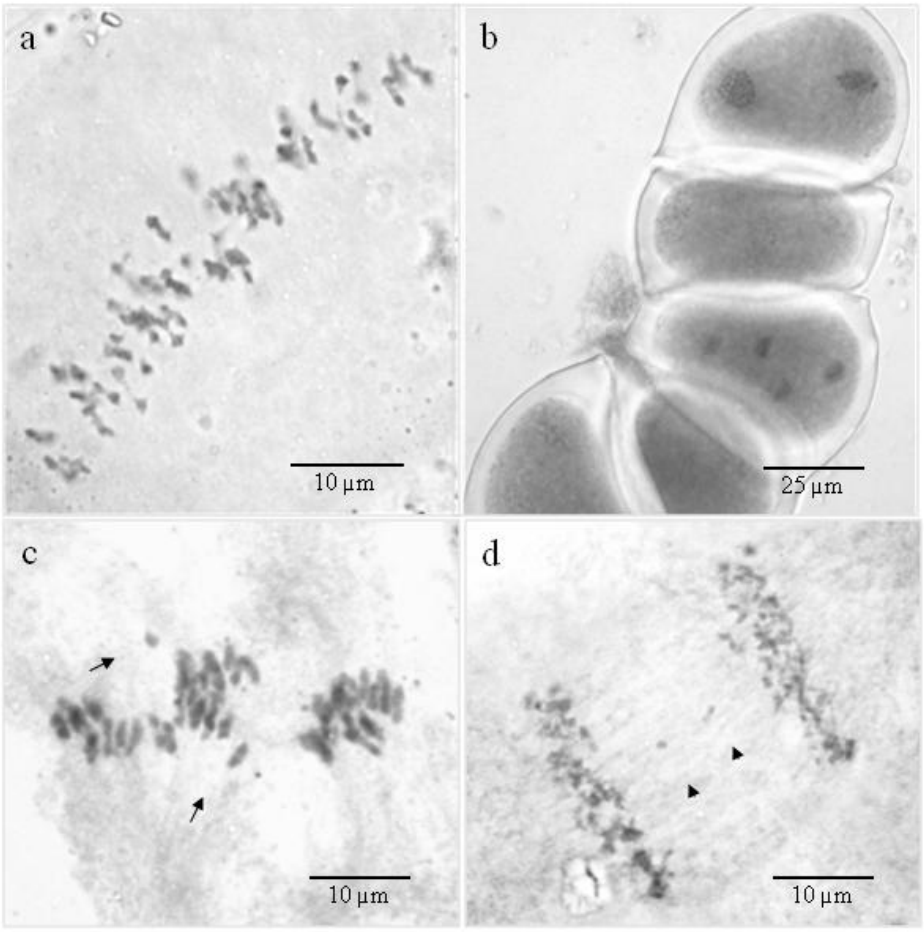

Fig. 2

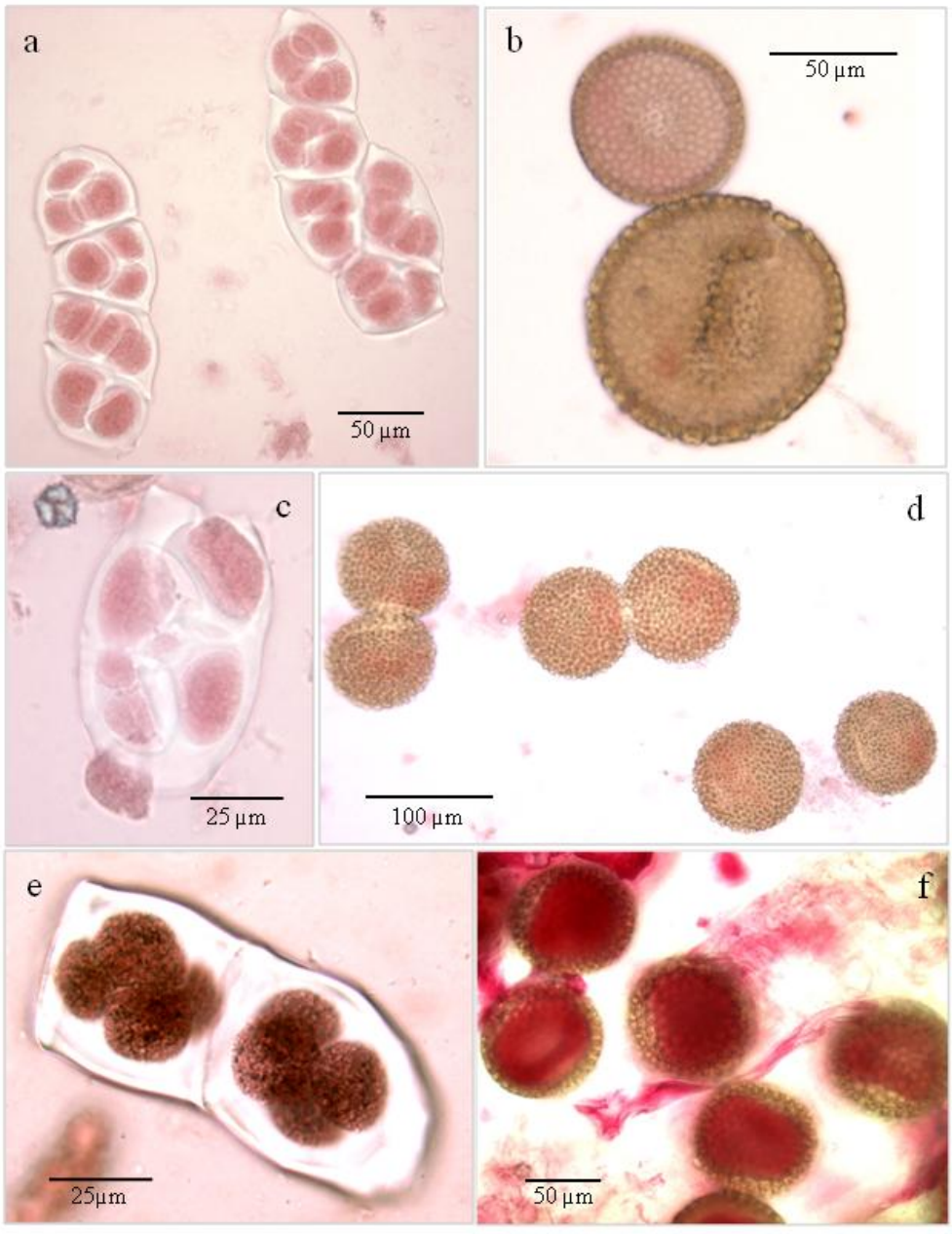

Fig. 3

CHAPTER IV

How do gap dynamics and colonization of a human disturbed area affect genetic diversity and structure of a pioneer tropical tree species?

ORIGINAL RESEARCH PAPER

How do gap dynamics and colonization of a human disturbed area affect genetic diversity and structure of a pioneer tropical tree species?

Article according to instructions of Forest Ecology and Management (submitted)

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ABSTRACT

Pioneer tree species exhibit life-cycle characteristics and population structures that are mainly affected by natural or human disturbances. In primary forests, demographic dynamics of pioneer species may resemble those defined for a metapopulation. In early successional forests, the patterns of establishment, survival and reproduction are mainly determined by microclimate and biota modifications of the site after the human disturbance. The aim of this study was to investigate how the ecological processes and the population genetic factors associated with disturbances can affect the genetic diversity and structure of populations of a pioneer tree species: *Croton floribundus* Spreng. (Euphorbiaceae). Nuclear and chloroplast microsatellite markers were examined in plants of two size classes sampled in four gaps of primary forest and four sub-areas of early successional forest. Despite presenting similar genetic diversity levels, the genetic diversity was distributed differently between forests. The combined effects of seed dispersal and colonizations (and extinctions) were determinants of the fine-scale genetic structure of *C. floribundus*. The main finding was that human disturbances seem to boost the influence of founder effects in populations of a species with limited seed dispersal. Results suggested that gene flow by pollen was responsible for maintaining the genetic diversity within populations of *C. floribundus* in both forests, but in the early successional forest, gene flow by seeds was equally important. We conclude that gap dynamics, colonization, and pollen and seed dispersal affect the genetic diversity and structure of the pioneer species depending mainly on the number of colonizers, the number of source populations, the gene flow rates, and the level of human disturbance.

Keywords: colonization, *Croton floribundus*, disturbances, forest succession, gap dynamics, genetic diversity, microevolution, pioneer tree species, semi-deciduous tropical forests.

1. Introduction

The spatial genetic structure of plant populations may vary across life stages, across generations and among different environmental conditions (Loveless and Hamrick, 1984). These changes are guided by ecological and evolutionary factors. Understanding the effects of these factors on the genetic diversity and structure of populations is important when defining strategies of forest conservation and restoration, in order to guarantee the maintenance of their evolutionary and ecological dynamics as similar as possible to the natural conditions (Loveless and Hamrick, 1984; Ellstrand and Elam, 1993).

Pioneer tropical tree species exhibit particular life histories and population structures that are mainly affected by the patchy dynamics of the tropical forests (Swaine and Whitmore, 1988; Alvarez-Buylla, 1994; Uriarte et al., 2012). The life-cycle characteristics of the pioneer species may have significant consequences for genetic structure and evolution, mainly through frequent local extinctions and colonizations (Wade and McCauley, 1988). We might expect clear population structuring in pioneer species because the number of gap colonizers is generally small (a founder effect), and due to genetic drift that leads to the fixation of neutral genetic alleles (Wright, 1940; Loveless and Hamrick, 1984; Whitlock and McCauley, 1990). Conversely, seed banks and long distance seed and pollen dispersal could homogenize spatial genetic variation (Loveless and Hamrick, 1984; Alvarez-Buylla and Garay, 1994). In addition to these factors, genetic divergence may also be reduced by extinction and recolonization processes which can act as a form of gene flow under some conditions of metapopulation dynamics. (Slatkin, 1977, 1985; Wade and McCauley, 1988; Whitlock and McCauley, 1990). Two opposite types of distribution of genetic diversity have been found in well conserved forests: nonstructured (Alvarez-Buylla et al., 1996; Davies et al., 2010) and genetically structured populations of adults (Jones and Hubbell, 2006; Born et al., 2008a). Nevertheless, most studies agreed with a finding of a high

level of genetic diversity of the populations and a loss of genetic structure across successive life stages.

In the early successional forests where environmental conditions differ from the primary forests (Swanson et al., 2011), the pioneer trees exhibit changes in their patterns of establishment, survival, and reproduction affecting plant density and spatial distribution (Silvestrini and Santos, 2014; Danciguer, 1996; Swanson et al., 2011; Tabarelli et al., 2012). Consequently, ecological processes and evolutionary factors, such as origin and number of colonizers, competition and predation, as well as natural selection and level of gene flow, either from primary or secondary forests, could be determinant of the genetic diversity and structure of the species.

There are few studies of genetic diversity and structure of pioneer species in early successional forests (Alvarez-Buylla and Garay, 1994; Franceschinelli and Kesseli, 1999; Cavers et al., 2005; Born et al., 2008b; Davies et al., 2010, 2013). The results have suggested that gene flow and the origin of populations are the determinants of the genetic patterns of diversity and structure that were found (Alvarez-Buylla and Garay, 1994; Franceschinelli and Kesseli, 1999; Cavers et al., 2005; Born et al., 2008b; Davies et al., 2010, 2013). However, in some of these studies, genetic differentiation has been evaluated simultaneously among old, intermediate, and recently established populations and/or sites with different land use histories, which according to genetic theoretical models is an uninformative way to estimate genetic structure in metapopulations (Wade and McCauley, 1988; Whitlock and McCauley, 1990; McCauley et al., 1995). As a result, the effects of ecological and genetic processes such as colonization, gene flow and level of disturbance might have been underestimated or blurred. Furthermore, only a few species have been evaluated and, most of these were restricted to colonizers of large gaps while the gaps are very open, i.e., a limited range of gap-phase regeneration behaviors (Brokaw, 1987) (for example, see Alvarez-Buylla et al. 1996 and Born et al. 2008b).

Croton floribundus Spreng. (Euphorbiaceae) is a shade intolerant, fast-growing pioneer tropical tree species (Lorenzi, 1992; Gandolfi et al., 1995) that is widespread and equally distributed along the whole gap-size gradient in primary forests, colonizes over extended time and is abundant in early successional forests (Silvestrini and Santos, 2014). This broad gap-phase regeneration behavior is particularly important for genetic studies because it allows us to analyze a species that occupies more heterogeneous environments, and thus, it is exposed to more diverse selection pressures. Besides this, *C. floribundus* does not seem to follow the same long-distance seed dispersal patterns of other genetically well-studied pioneer species that have shown high gene flow rates (Alvarez-Buylla and Garay, 1994; Litrico et al., 2005; Born et al., 2008a). *C. floribundus* is pollinated by flies and wind (Passos, 1995), which implies a long distance pollen dispersal and high rates of gene flow (Hamrick and Nason, 2000), but has seeds dispersed short distances by autochory (explosive seed dispersal) (Lorenzi, 1992). Thus, a fine-scale study of genetic diversity and structure of this species using both nuclear and chloroplast markers may help to elucidate how these mechanisms of dispersal, with opposite effects on gene flow rates, affect the distribution of the genetic variation in its populations. Analysis of both nuclear and chloroplast markers can also be helpful for investigating effects of the colonization process on the genetic structure among populations by means of cyto-nuclear linkage disequilibrium estimates (Fields et al., 2014).

In addition, a comparison between populations located in primary and early successional forests and a detailed analysis of the fine-scale genetic structure among life stages or size classes could shed light on what factors determine the patterns of genetic diversity and structure in this species. The knowledge of such aspects of pioneer species is essential for understanding the effects of human disturbance and of successional processes in tropical forests, which in turn is important for forest conservation, management and restoration.

In this study, we evaluated the genetic diversity and structure of the pioneer tree *C. floribundus* between two size classes and among populations located in a primary forest and in an early successional Brazilian forest. Specifically, we attempted to answer the following questions: 1) Do the genetic diversity and fine-scale genetic structure of *C. floribundus* differ between primary and early successional forests? 2) Are there changes in the genetic diversity and structure between size classes in these forests? 3) Is there a correspondence between the genetic structure of populations in the primary forest and their spatial distribution in the gaps? 4) How do ecological processes and genetic factors associated with natural and human disturbances affect the genetic diversity within and among populations in the species?

2. Materials and methods

2.1. Study site

The study was conducted within a 10.24 ha permanent plot (Rodrigues, 2005) in the primary forest at Caetetus Ecological Station (CES) and in an early successional forest adjacent to the state reserve at Torrão de Ouro Farm, Gália/Alvinlândia, Brazil (Fig. 1) (22°20' - 22°30'S; 49°40' - 49°45'W). The regional climate corresponds to Köppen's "Cwa" mesothermic type, that is, humid subtropical with a dry winter (Tabanez et al., 2005; Rodrigues, 2005). Mean rainfall and temperature are 1 431 $mm.yr^{-1}$ and 21.5 ºC, respectively. Altitude ranges from 520 to 680 m (Tabanez et al., 2005). CES is a large remnant of the semi-deciduous tropical forest of 2 178.84 ha, which corresponds to the premontane moist forest (Holdridge, 1967). According to historical reports dating from the early 20^{th} century, most of the area including the center of the reserve, where the 10.24 ha permanent plot was delimited, has not experienced anthropogenic disturbance (Tabanez et al., 2005). The land-use history of the selected site at Torrão de Ouro Farm revealed that the pristine forest was cleared in 1926 and converted to a pasture. In 1984, the site was

abandoned and farmers stopped slashing, but cattle grazing was still allowed. In 1986, the vegetation was burned, and in 2008, the pasture was completely closed to cattle grazing, allowing the forest to recover. Sampling for this study was carried out in 2010.

Soils in the early successional forest at Torrão de Ouro Farm are of the same type as at the CES plot, i.e., the red-yellow Acrisols (Ultisols), but at the E' sub-area (see Sampling for details), soils are Gleysols (Entisols) with two different physical textures (M Cooper, Personal communication; Electronic Supplementary Material, Tables A1 and A2). Soil fertility is high and nearly similar between forests (Rodrigues, 2005; M Cooper, Personal communication; Electronic Supplementary Material, Tables A1 and A2).

2.2. Study species

Croton floribundus Spreng. (Euphorbiaceae) is a shade-intolerant, fast-growing pioneer tree species commonly found in gaps of primary remnants and in secondary areas of the semi-deciduous tropical forest (Lorenzi, 1992; Gandolfi et al., 1995; Rodrigues, 2005). Tree height ranges from 4 to 13 m in open habitats (Lorenzi, 1992; Danciguer, 1996), but in the primary forest at CES, tree height ranges from 3 to 30 m (Rodrigues, 2005; TE Barreto, Unpublished results).

The seeds require alternating temperatures for germination in light as well as in darkness (Válio and Scarpa, 2001). Seed dispersal occurs by autochory or ballochory, i.e., plants disperse their offspring ballistically, by the explosive opening of the fruits (explosive seed dispersal) (Lorenzi, 1992). We assumed that *C. floribundus* does not persist in the soil seed bank because the seeds are heavily attacked by predators (Carvalho 2001) and present a short period of viability (Lorenzi, 1992).

C. floribundus is a polyploid (2n=8x=112), self-compatible, and monoecious species (Silvestrini et al., 2013; Passos, 1995). The flowers can be pollinated by flies and wind, Sirphid flies being the most important pollen vectors in *C. floribundus* (Passos, 1995). Dichogamy is much accentuated and seems to reduce self-fertilization in *C. floribundus*. The species also presents agamospermy, although this produces fruits with inviable seeds (Passos, 1995).

2.3. Sampling

Individuals of two size-classes were sampled. The selection of the two size classes was based on Danciguer's (1996) definition of life stages for *C. floribundus* and a previous evaluation of *C. floribundus*' distribution in the gaps of the permanent plot (Silvestrini and Santos, 2014). Juveniles consisted of individuals of 5 to 37 cm height and 0.05 to 0.40 cm diameter at soil height (DSH) (Danciguer, 1996). Adults comprised individuals of 4.78 to 20.00 cm diameter at breast height (DBH) , although they are not necessarily reproductives (Silvestrini and Santos, 2014). The definition of the adult size class was also based on the criterion of inclusion of trees in the plant inventory in the permanent plot (DBH $\geq$ 4.78 cm) (Rodrigues, 2005).

Since *C. floribundus* is widespread and equally distributed along the gap-size gradient, as well as the range of gap ages in the primary forest (Silvestrini and Santos, 2014), we sampled juveniles in four gaps with different characteristics (size, age, modes of disturbance) in order to evaluate the genetic structure across the gap heterogeneity. The canopy gaps were named according to the subplot where they were located (Fig. 2, Table 1). Also, a pairwise sampling between juveniles and adults, i.e., adult gaps adjacent to juvenile gaps, was used (Fig. 2, Table 1). This sampling design enabled us to examine the possible parentage relationships between adults and juveniles by increasing the probability of sampling mother trees and their offspring over short distances. In total, sixty-four (64) juveniles and forty-nine (49) adults were tagged,

sampled, measured for DBH (adults), height and DSH (juveniles) (Table 1). In order to facilitate nomenclature and comparisons with the early successional forest, the same letter hereafter is used to identify juveniles and adults of adjacent gaps.

The sampling design in the early successional forest at Torrão de Ouro Farm was as similar as possible to CES. The forest fragment was subdivided into four sub-areas. The distances between each pair of sub-areas corresponded to the counterpart pair of gaps at CES (Fig. 2). Populations of juveniles were delimited in the sub-areas based on the gap area of juveniles in CES. Hence, an area about 243 m^2 for C', 950 m^2 for E', 292 m^2 for K', and 514 m^2 for M' was censused in order to find and select the study individuals. For adults, we selected individuals with a similar spatial distribution to the counterpart population of adults in the gaps of CES (equivalent perimeter and area covered by sampled trees and similar distances between trees). Sixty-four (64) juveniles and forty-eight (48) *C. floribundus* adults distributed in the four sub-areas (Fig. 2) were tagged, sampled, measured for DBH (adults), height and DSH (juveniles). The number of individuals sampled per sub-area (N) was 16, 18, 14, 16 for juveniles and 11, 11, 13, 13 for adults in the C', E', K' and M' sub-areas, respectively.

All sampled individuals had their geographic coordinates recorded and were mapped using GIS software ArcView® (version 9.2, ESRI, Redlands, California, USA).

Two to three young leaves per juvenile plant were collected, frozen in liquid nitrogen and stored at -80^{o}C for DNA extraction. For adults, leaves were frozen at -20^{o}C and stored in sealed paper bags for as much as three weeks until maceration.

2.4. Microsatellite markers

Seven nuclear microsatellite loci (SSR) developed for *C. floribundus* (Silvestrini et al., 2013) and six universal chloroplast microsatellite (cpSSR) loci obtained from Weising and

Gardner (1999) and optimized for *C. floribundus* (Silvestrini, 2014) were used to study the genetic diversity and structure of the species. Total (genomic and chloroplast) DNA was extracted from young leaves according to Doyle and Doyle (1990), using CTAB as a detergent. DNA quality and quantity was checked on agarose (0.8% w/v) gels stained with SYBR Safe® (Invitrogen Corporation, Carlsbad, California, USA) by comparison to lambda (λ) DNA standards. All DNA samples were diluted to a final concentration of 20 $ng.\mu L^{-1}$.

PCR amplifications of both nuclear and chloroplast microsatellites were performed according to Silvestrini et al. (2013). Amplification products obtained using 5' end fluorescent-dye-labeled forward primers (6-FAM and 6-HEX), were analyzed for size on the automated sequencer 3730 DNA Analyzer (Applied Biosystems, Inc., Foster City, California, USA). SSR and cpSSR loci were scored using Peak Scanner software v1.0 (Applied Biosystems, Inc.). Multiplex genotyping was performed for chloroplast markers (two cpSSR loci per line).

2.5. Data Analysis

2.5.1. Genetic diversity

SSR loci were scored by presence or absence of bands or alleles. As *C. floribundus* is polyploid with unknown pedigree and likely disomic inheritance (Silvestrini et al., 2013), we cannot distinguish nuclear genotypes and estimate allelic frequencies by standard methods or use computer programs that allow allele copy ambiguity under the assumption of autosomic inheritance. Thus, allelic frequencies (p_i) were estimated as the relative frequency of the j^{th} allele or band for each SSR marker in the population. For genetic structure analyses and some genetic diversity estimates such as proportion of polymorphic loci (*P'*), gene diversity (*H'*), and Shannon's genetic diversity index ($H'_{Shannon}$), however, the SSR markers were treated as

multilocus fingerprints, in which each allele was either scored present or absent (p_i'). Monomorphic bands or alleles were included in all analyses in order to allow invariant loci to influence diversity values.

Patterns of genetic diversity were examined for each group of individuals of the same size class in the same gap or sub-area for the primary forest and for the early successional forest, respectively. This individual grouping is hereafter referred to as a "population". The following genetic parameters: effective number of alleles per locus (A_e), gene diversity (H and H'), and Shannon's genetic index ($H'_{Shannon}$) were estimated for the nuclear markers according to Silvestrini et al. (2013). Average number of alleles per locus (A) was determined by summing all the alleles observed and dividing by the total number of loci. Proportion of polymorphic loci was calculated by dividing the number of polymorphic loci by the total number of loci analyzed and was calculated considering the seven SSR loci (P) and each allele/band as a locus (P'). Allelic richness (R) was estimated as the number of different alleles per population standardized for the smallest sample size (considering the missing data) (N=9) using the rarefaction method of Petit et al. (1998) implemented in the software CONTRIB. The number of private alleles (A_p), i.e., alleles that occur in only one population, was counted for all populations. A_p was also counted for both forests as the number of alleles that occur in only one forest type. The rarefaction procedure was not applied to the number of private alleles (A_p) as the methodology has not been developed for polyploids. As the number of private alleles is positively dependent to the number of alleles per locus (Kalinowski, 2004), we also calculated the ratio of private alleles over the average number of alleles per locus (A_p/A). For estimating A_p/A ratio in each forest, we used the mean of A values calculated over eight populations as the denominator.

Each unique combination of alleles of all cpSSR loci was defined as a haplotype. The cpSSR haplotype frequencies were calculated for all populations (p_i) and used to calculate

number of haplotypes (*n*), haplotype richness (*r*), number of private haplotypes per population (n_p), and haplotype diversity (*h*). *r* was estimated as the number of different haplotypes per population standardized for the smallest sample size (considering the missing data) (N=8) using the software CONTRIB (Petit et al., 1998). *h* was calculated as the probability that two randomly chosen haplotypes are different in the sample according to Nei (1987):

$$h = \frac{n}{n-1}\left(1 - \sum_{i=1}^{k} p_i^{\,2}\right)$$

where n is the number of individuals in the sample, k is the number of haplotypes, and p_i is the sample frequency of the *i*-th haplotype.

Genetic diversity estimates for both nuclear (*A*, A_e, *P'*, *R*, A_p, A_p/A, *H*, *H'*, $H'_{Shannon}$) and chloroplast (*n*, *r*, n_p, *h*) markers were analyzed using a two-factor ANOVA. The factors were forest type and size-class. Analyses were performed using Systat 11 software (Systat Software Inc., Richmond, California, USA).

2.5.2. Genetic structure

Population genetic structure for both classes of DNA marker was inferred by analysis of molecular variance (AMOVA - Excoffier et al., 1992) using Arlequin V. 3.5 software (Excoffier and Lischer, 2010). A hierarchical sampling design was employed using different groups of populations. First, fixation indices were estimated for each forest type independently by grouping 1) populations by size class, 2) populations from each size class separately, and 3) all populations without hierarchy. Second, populations from both forest types were analyzed together (all populations). Partitioning of the genetic diversity was evaluated 1) between size classes, 2) between forests for each size class separately, and 3) between forests. The significance of the

fixation indices obtained (i.e., whether values are different from 0) was tested by using a non-parametric permutation approach (Excoffier et al., 1992) for 1 000 permutations. A locus by locus AMOVA, i.e., the AMOVA for each locus separately, was performed for nuclear markers. Confidence limits of the F_{ST} indices were obtained by bootstrapping over loci (20 000 bootstraps).

A Bayesian cluster analysis implemented in the software Structure version 2.3.3 (Pritchard et al., 2000; Falush et al., 2007) based on haploid data (presence or absence of bands or alleles), was employed to evaluate genetic structure of individuals on different scales of distribution of populations. Assuming the admixture model and correlated allele frequencies, 20 independent runs were performed for each K (a priori number of clusters) (K=1 to K=16) at 500 000 MCMC repetitions and a burn-in period of 200 000. Using the program Structure Harvester (Earl and vonHoldt, 2012), the approach described by Evanno et al. (2005) was adopted to define the most reasonable K by using DeltaK as the criterion. A mean of the permuted matrices across the 20 replicates was obtained for the two most likely numbers of clusters (K) employing the program CLUMPP (Jakobsson and Rosenberg, 2007) and visualized through the program DISTRUCT (Rosenberg, 2004).

To assess spatial autocorrelation and fine-scale spatial genetic structure, genetic distograms and correlograms were constructed for SSR and cpSSR data sets, respectively, using the program SGS (Degen et al., 2001). The genetic dissimilarity was computed using Tanimoto's distance as described by Degen (2000). For cpSSR haplotypes, correlograms were computed using Moran's index (Degen, 2000). Minimum haplotype frequency of 0.05 was used as an inclusion criterion in the calculations.

Spatial autocorrelation was examined among all sampled individuals of each forest type in order to evaluate fine-scale genetic structure and the genetic relationships between adults and

juveniles, and among individuals of each size class separately. The mean genetic dissimilarities and Moran's index were calculated for eight different spatial distance classes of 30 m each from 0 to 240 m when analyzing all individuals and seven different spatial distance classes of 40 m each from 0 to 280 m when analyzing each size class independently. The sizes of 30 and 40 m for each spatial distance class were chosen so that in all cases at least 30 pairs of plants belonged to each class. Different distance classes (17 classes of 15 m, ten classes of 25 m, nine classes of 28 m, and six classes of 45 m each) were employed whenever possible in order to check the influence of distance class definition on the results, as suggested by Degen et al. (2001).

The effect of the extinctions and colonizations on genetic structure was evaluated by estimating the number of colonists (k-values) of the recently founded populations and applying the estimates to the general model of founding group formation of Whitlock and McCauley (1990) with slight modifications in order to accommodate the polyploid nature of the data and obtain the probability of common origin of gametes (ϕ):

$$F_{ST0} = 1/k + \phi(1 - 1/k)(F_{STc})$$

where k is the number of individuals that comprise a colonizing group, F_{ST0} is the F_{ST} among only the new populations and F_{STc} is the F_{ST} defined among established populations. The symbol ϕ is defined as a probability that two alleles in newly formed population were drawn from the same source, which varies from 0 to 1 (Slatkin, 1977; Wade and McCauley, 1988; Whitlock and McCauley, 1990). For the purpose of this study two time and spatial scales of colonization were investigated: 1) colonization of the recent founded populations of gaps in the primary forest (juveniles) by colonists from the older established gaps (adults); and 2) colonization of the early successional forest (adults) by colonists from the established populations of the primary forest and surrounding secondary forests. As we do not have any genetic structure estimate from the

adjacent secondary forests in different stages of succession (early, intermediate, late successional stages) or the ones at the boundaries of CES, we used the F_{ST} estimated from all adult populations of both forest types as the approximate F_{STc} values of old established populations.

For the first approach, k-values were estimated based on the number of juveniles of *C. floribundus* recorded in the four gaps (Table 1A). For colonization of the early successional forest, colonists were estimated as the number of the individuals first established in the area, i.e., the number of adults in the sub-areas. As the sampling of populations in the early successional forest was based on an approximation of the gap areas in the primary, the number of colonists was obtained multiplying the density of adults estimated for the early successional forest (Silvestrini and Santos, 2014) by the total area of gaps (Table 1B).

2.5.3. Linkage disequilibrium between nuclear and chloroplast alleles

Cyto-nuclear linkage disequilibrium between SSR alleles and cpSSR haplotypes (D) was calculated for all possible pairs of the most frequent alleles of each SSR locus (p_i') and the three commonest cpSSR haplotypes after pooling all individuals sampled from the early successional forest using the equation described by Hedrick (2011). Afterwards, the linkage disequilibrium (D) value was standardized to the maximum value possible (D') (Hedrick, 2011) and the statistical association for each pair of allele/haplotype was tested using G-tests of independence (α =0.05) (Sokal and Rohlf, 2012), the same method used by McCauley and Ellis (2008) and Fields et al. (2014). The overall family-wide error rate was corrected using a Bonferroni method (Sokal and Rohlf, 2012) by employing a critical value of $\alpha'' = 0.0015$.

3. Results

3.1. Genetic diversity

We found 77 alleles or bands through amplification of seven SSR loci in 225 individuals of *C. floribundus*, considering both forest types (Electronic Supplementary Material, Table A3). Only two bands were monomorphic for all populations, one from locus SSR-8 and the other from SSR-6. All SSR loci were polymorphic for all populations studied (*P*). Genetic diversity estimates did not differ statistically between forest types and size classes, except for *P'* (p=0.011) and *A* (p=0.011), which have higher values for juveniles than adults in both forests (Table 2, Electronic Supplementary Material, Fig. A1). However, after rarefaction that standardizes the number of alleles across unequal-sized populations, *R* values did not significantly differ between size-class (p=0.096) (Table 2).

Despite similar values of A_p and A_p/A between forests (p=0.230 and p=0.304, respectively) (Table 2), when the number of private alleles (A_p) was counted as alleles that occur in only one forest type, A_p and A_p/A values were 13 and 1.86, respectively in the primary forest, and 6 and 0.92, respectively, in the early successional forest.

Of six cpSSR loci studied, only three showed polymorphisms in *C. floribundus* and only in the populations of the early successional forest (Table 3 and Electronic Supplementary Material, Table A4). In total, seven haplotypes were detected: one in the CES and seven in the early successional forest (Fig. 3 and Electronic Supplementary Material, Fig. A2). The number of haplotypes (*n*), haplotype richness (*r*), and haplotype diversity (*h*) were statistically higher in juveniles than adults ($p<0.01$) and in early successional forest than primary forest ($p<0.001$) (Table 3). The relative frequency of haplotypes varied among populations (Table 3, Fig. 3).

3.2. Genetic structure

Despite presenting similar genetic diversity levels, the genetic diversity was distributed differently between primary and early successional forests (Table 4). Differentiation between size

classes within gaps (F_{SC}=0.083, p<0.0001) showed that allelic frequencies are differently distributed between juveniles and adults from the "same gap". Likewise, F_{SC} between juveniles and adults within sub-areas in the early successional forest was different from zero (F_{SC}=0.060, p<0.0001) (Table 4). Adults presented a stronger differentiation among populations than juveniles. There was overlap of the confidence intervals between F_{ST} values of juveniles and adults in the primary forest, but with a tendency of greater divergence among populations of adults (Fig. 4). This tendency was confirmed by results of Bayesian structure, and spatial autocorrelation analyses (see below).

When considering the cpSSR markers, the differentiation between juveniles and adults within sub-areas in the early successional forest was high and different from zero (F_{SC}=0.363, p<0.0001), corroborating the dissimilar distribution of haplotype frequencies between size classes of the same sub-area (Table 4, Fig. 3B). Higher genetic subdivision among populations of adults (F_{ST}=0.653, p<0.0001) than juveniles (F_{ST}=0.328, p<0.0001) was found.

The Bayesian structure analysis of *C. floribundus* in both forest types discriminated the individuals into three and eight clusters. After 20 repeated runs for K numbered from 1 to 16, DeltaK reached the highest peak at K=3. The first cluster was comprised basically of individuals from primary forest, the second individuals from the early successional forest and the third individuals from sub-area K' (Fig. 5A). An additional but a smaller peak was observed at K=8. This clustering was complementary to the K=3 allowing one to distinguish individuals of gap M from other gaps in the primary forest as well as individuals of each sub-area in the early successional forest (Fig. 5B).

A positive autocorrelation was detected in the first class of 15 m for individuals of the primary forest (Fig. 6A) and in the distance class of 30 m for the early successional forest (Fig. 6B), for it was not possible to use shorter distance classes in the latter analysis. Despite this,

results of Structure analysis and cpSSR-haplotype frequencies indicate that, similar to the primary forest, positive spatial autocorrelation can also occur in shorter distances in the early successional forest. In both forest types, there was a negative spatial autocorrelation from 30 to 60 m (Fig. 6A,B). In the primary forest, these distance classes corresponded to pairwise distances between juveniles and adults of the "same gap" and some pairs of individuals within size classes at K and M gaps (mainly adults, see Fig. 2). In the early successional forest, these distances comprised individuals at sub-areas K' and M' and some individuals at sub-area E'. Note that juveniles and adults in the primary forest occupy different gaps (Silvestrini and Santos, 2014), although we have considered them as belonging to the "same gap", while juveniles and adults in the early successional forest in fact occupy the same sub-area. For cpSSR markers, the correlogram presented a similar autocorrelation pattern to the distogram until 90 m (Fig. 6C).

The distogram plotting genetic distance against spatial distance class for each size class separately showed individuals closely related within a 40 m distance in both forest types (Fig. 7A-D). In contrast to juveniles, adults presented a significant negative spatial autocorrelation in the second distance class (40 to 80 m) and in the sixth distance class (200 to 240 m) in the primary forest, demonstrating a higher divergence among populations in this size class (Fig. 2 and Fig. 7A,B). In the early successional forest, random or negative spatial genetic structures were found in distances >40 m for both size classes (Fig. 7C,D).

Higher values of the Moran's coefficients than expected indicated a positive spatial autocorrelation for cpSSR markers for juveniles and adults in the early successional forest within the distance class of 40 m (Fig. 7E,F). A significant negative spatial autocorrelation was observed at other distance classes (Fig. 7E,F), which corroborated the divergent distribution of haplotype frequencies among populations in the early successional forest (Fig. 2 and Fig. 3, Table 4).

The number of colonists (k) estimated for colonization of the recently founded populations of gaps in the primary forest (juveniles) was k=69. For colonization of the early successional forest (adults), k=9. The ϕ estimates for these new populations were 0.49 and ≃ 1.0 for primary and early successional forest, respectively.

3.3. Linkage disequilibrium between nuclear and chloroplast alleles

Considering the small sample size (N=112) in cyto-nuclear linkage disequilibrium analysis and the magnitude of the D' values found (Electronic Supplementary Material, Table A5), cyto-nuclear linkage disequilibrium was an important phenomenon in populations of *C. floribundus*. The strong negative association between allele SSR-6_205 and haplotypes 1 (D'=-1, p=0.0163) and 2 (D'=-1, p=0.0004) as well as the positive association with haplotype 5 (D'=0.292, p=0.0073) showed that there are higher frequencies of allele SSR-6_205 in early successional forest populations in which haplotype 5 is the most frequent, i.e., in the sub-areas C' and M' (see Fig. 3B). Also, the allele SSR-16_211 was positively associated with haplotype 5 (D'=0.746, p<0.0001), showing a higher frequency in the same sub-areas.

4. Discussion

A moderate number of alleles per locus and effective number of alleles per locus, as well as high genetic diversity indices, were found when compared to other tropical tree species (Lemes et al., 2003; Degen et al., 2004; Ndiade-Bourobou et al., 2010; Cavallari et al., 2010), indicating a high genetic diversity within populations of *C. floribundus* in both forest types. Indeed, high genetic diversity levels have been commonly found for pioneer tree species (Alvarez-Buylla et al., 1996; Alvarez-Buylla and Garay, 1994; Litrico et al., 2005; Jones and

Hubbell, 2006; Born et al., 2008a, b; Davies et al., 2010) but the great majority of prior research has evaluated pioneer species that regenerate only in large gaps. Also, only a few studies have evaluated genetic diversity between early- and late-successional populations and they either considered primary succession (Litrico et al., 2005) or areas with different or unspecified land use histories (Alvarez-Buylla and Garay, 1994; Cavers et al., 2005; Born et al., 2008a). Only Davies et al. (2010) have found a significant reduction in genetic diversity between populations in an old growth forest (no history of human land use) and a secondary forest stand about 30 years in age. The reason for the genetic bottleneck found was that there were limited sources for seed dispersal and colonization (Davies et al., 2010, 2013). To our knowledge, this is one of few studies that compares genetic diversity and structure of a pioneer species among populations in a primary forest and in an early successional forest where populations experienced similar and well-known human disturbances.

The close relationships among individuals within populations and among juveniles and adults of the "same gap" or sub-area over short distances (up to 30 m) indicated a high level of relatedness, perhaps of siblings, half-siblings and/or parents and offspring. This genetic similarity is likely a result of the explosive or ballistic seed dispersal mechanism of the species that results in average dispersal distances of 1.5 to 3 m (Stamp and Lucas, 1983; Narbona et al., 2005) or approximately 3.2 and 3.4 m (mean ballistic dispersal distance) plus 1.0 to 2.5 m (distance of seed removal by ants) for the autochorous tree species with secondary dispersal via myrmecochory, *Croton priscus* Croizat (Passos and Ferreira, 1996). On the other hand, the negative spatial autocorrelation in the distance class of 45 m in the primary forest, which corresponds mainly to the distances between juveniles and adults of the "same gap", confirmed the genetic dissimilarity between juveniles and adults within gaps. Also, the genetic differentiation between adults and juveniles of the same sub-area was high. These contrasting

results revealed that seed dispersal has an effect on the genetic similarity of juveniles and adults due to likely parent-offspring proximity, though at the same time different colonization events have an opposite effect producing marked genetic differences between adjacent pairs of juveniles and adults. Also, the effects of aggregated seed dispersal, i.e., the genetic structure given by offspring-offspring proximity, seem to be essential for the formation of the kin-structure observed within populations.

According to Wade and McCauley (1988), Whitlock and McCauley (1990) and McCauley et al. (1995), the genetic structure of recently colonized populations depends mostly on the number of individuals involved in the founding event (k), the relationship between number of colonists and migrants, and the number of source populations from which the colonists were drawn. The model of Wade and McCauley (1988) predicts that "whenever the number of new colonists is less than twice the number of migrants, extinctions and recolonizations always diminish effective gene flow and enhance genetic differentiation of subdivided populations". In our sampled gaps in the primary forest, the number of colonists seems to be much greater than number of migrants since adjacent adult populations increase the input of seeds. The average gene flow in the whole gap area distribution, however, may not reach such high levels because only some of the recently colonized gaps are adjacent to the old ones occupied by adults of *C. floribundus* (Silvestrini and Santos, 2014). In addition, the presence of large trees and dense tangle of lianas in the primary forest (Rodrigues, 2005; Lima et al., 2008) must be an effective barrier to seed arrival in the open gaps, given the absence of cpDNA haplotype diversity for both size classes. Thus, it seems that the high input of seeds into the study gaps contributed to the high number of colonists and the consequent low genetic structure in juveniles. Open areas near adult populations may also facilitate wind pollination and as a consequence promote an increase in the number of colonists in juvenile populations by increasing the diversity of pollen gametes in the

fertilized seeds. Additionally, since pollen dispersal by wind and insects/flies can reach long-distances, over several hundred metres (Hamrick and Nason, 2000), it seems that pollen came from more diverse sources than the female genetic material of the founder seeds.

In effect, our ϕ estimates in the primary forest indicated an intermediate form of colonization between the propagule and migrant pool ($\phi = 0.49$) (Slatkin, 1977; Wade and McCauley, 1988; Whitlock and McCauley, 1990) suggesting that colonists, mostly by pollen in fertilized seeds, originated in more than one population. In the complete propagule model, extinctions and colonizations are always expected to increase genetic structure of new populations, particularly when the number of colonists is low (Wade and McCauley, 1988). However, in migrant pool models, when the number of colonists exceeds twice the number of migrants, mainly under low extinction rates, extinctions and colonizations can act as form of gene flow as predicted by Slatkin (1977; 1985; 1987) and reduce genetic structure (Wade and McCauley, 1988). As we mentioned above, the number of colonists in the juvenile gaps must be higher than the number of migrants. In addition, *C. floribundus* has long-term population persistence in the primary forest (Silvestrini and Santos, 2014), which may indicate a low extinction rate for *C. floribundus* populations. Therefore, under an intermediate mode of colonization, at least a certain amount of gene flow might be generated as a result of extinctions and recolonizations.

Conversely, the extended life-time of populations may allow other processes such as selection, drift and inbreeding to act. These factors are not included in the theoretical models (Wade and McCauley, 1988; Whitlock and McCauley, 1990) and may help to explain the genetic differentiation among populations in the primary forest, especially for adults. Although juveniles of *C. floribundus* usually do not occupy the same gap as adults, juveniles were found in adult gaps that suffered recent recurrent disturbances (Silvestrini and Santos, 2014). Thus, there might

to be an effect of the random sampling of gametes in the gaps (genetic drift) and mating between relatives, particularly if the recurrent episodes are frequent, which seems to happen in the CES permanent plot (Lima et al., 2008), and if the generations immediately succeeding the recurrent disturbance rapidly reach the reproductive life stages (Silvestrini and Santos, 2014).

In contrast to other pioneer species (Alvarez-Buylla et al., 1996; Jones and Hubbell, 2006; Born et al., 2008a, b), *C. floribundus* exhibited an increase in genetic structure across successive life stages in both forest types. We believe that our study design, which distinguished human disturbed populations from undisturbed ones and analyzed adults distributed in gaps, allowed us to distinguish the effects of human disturbances and gap dynamics on genetic diversity and structure and revealed how these factors really affect the genetic structure in different size classes. In addition, this seems to be the first genetic study of a pioneer species that has a larger gap-phase regeneration behavior (Brokaw, 1987; Silvestrini and Santos, 2014). The occupation of a more heterogeneous gap environment may also contribute to the divergence among populations found in the primary forest, mainly in adults.

In the early successional forest, our results suggest that the greater distances separating the vacant sites from the boundaries of the old populations and the inbreeding generated by the movement of individuals or genes in groups, as with full-or half-sib seeds, were likely causes of our observed reduced effective number and a stronger kin-structure of populations of colonists (Wade and McCauley, 1988; Whitlock and McCauley, 1990) (see also results of Alvarez-Buylla and Garay (1994) and Born et al. (2008b) for long-distance seed dispersal species whose pristine and disturbed populations were paired next to each other). The reduction in the number of colonists relative to gene flow might also be a consequence of some barriers to seedling establishment in the human disturbed area, such as cattle grazing and competition with grasses and weeds (Uhl et al., 1991; Holl et al., 2000). Note that the land use history of the early

successional study forest shows that propagules for colonization must come from external sources since the area was intensively used as pasture for many years followed by burning. Moreover, the history of land use and general environmental conditions were similar for all sub-areas, thus genetic differentiation is more likely the result of colonization process rather than specific characteristics of the sub-areas affecting plant survival and growth. Even with the low number of colonists, the higher number of cpSSR haplotypes for adults in the early successional forest showed that the arrival of seeds in this open habitat is higher than in the gaps of the primary forest, confirming that trees and lianas must be an effective barrier to seed dispersal in the primary forest. Also, it seems that there is a higher cpDNA diversity in the source populations that colonized this forest since it is surrounded by a mosaic of secondary forests with different land use histories.

In the next generations, however, the factors that reduced number of colonists seem to change as suggested by the reduction of both F_{ST} for nuclear and cpSSR markers and the increase of haplotype richness and diversity in juveniles. Besides the reasons mentioned above for adults, the high number and diversity of haplotypes in juvenile populations could be the effect of the high density of juveniles in the early successional forest (Silvestrini and Santos, 2014). This, in turn, is a result of the increase in gene flow by seeds, slow growth, and accumulation of plants from multiple events of seed dispersal due to the unfavorable growth conditions in this life stage (Danciguer, 1996; Silvestrini and Santos, 2014). Thus, across generations, the founding effects and barriers to plant establishment tend to be reduced and gene flow by seeds and pollen may become the main determinants of the genetic structure.

Apparently, the number of pollinators of *C. floribundus* is not a limiting factor for colonization or gene flow by pollen in the early successional forest. Beside the fact that the open area facilitates wind-pollination, richness and abundance of flies of Sirphidae family were greater

at the edge than inside the forests (Marinoni et al., 2004). This indicates that both of the most important pollen vectors of *C. floribundus* are abundant at that location. Indeed, high pollen flow rates have been typically found for tropical trees in fragmented or disturbed landscapes (Hamrick, 2004; Wang et al., 2011), including pioneer tree species (Born et al., 2008a; Davies et al., 2013). Thus, effective pollen movement might maintain the level of genetic diversity within populations during colonization (high variability of the pollen that fertilized founder seeds) as well as increase gene flow and its genetic homogenizing effects in the following generations.

Cyto-nuclear linkage disequilibrium is only expected in recently established populations if there is a genetic structure in both markers in the old established or original source populations (Fields et al., 2014). The reason is that the LD in the new populations reflects the earlier structure in the older because there has not been enough time for evolutionary forces to act on the allele and haplotype frequencies in the recently founded populations. Thus, as we found a genetic structure for nuclear markers but did not detect any variation in cpSSR markers in the sampled populations of the primary forest, our results indicated that there is a genetic structure at a greater spatial scale for the cpSSR loci in the primary forest or in the surrounding secondary forests and that colonists of the early successional forest may have come from these divergent populations.

Results based on private SSR alleles in both forest types, cpSSR haplotypes, assignment tests, and the genetic relationship among individuals confirmed that the origin of the early successional forest is not mainly from the primary forest, or at least not from our sampled populations from the primary forest. The limited seed dispersal of the species and the genetically structured populations in the gaps of the primary forest must keep different alleles widespread over the primary forest that we could not capture by our sampling. Thus, it is much likely that founders have come from primary populations spatially closer to the early successional forest

area and/or from the neighboring secondary forests at the edges of the reserve and at the Torrão de Ouro Farm.

In spite of the uncertain ancestry of the early successional forest alleles, it is a reality that for conservation purposes, genetic diversity of both forest types should be maintained as long as they harbor singular allelic richness. In a scenario of an almost complete devastation of the primary Brazilian Atlantic forests (Ribeiro et al., 2009) and the consequent absence of such genetic pools, secondary forests are the foremost remnants of genetic diversity of the species and therefore should be conserved whatever the successional stage.

So far there has been little concern for conservation of genetic resources of pioneer species due to the high genetic diversity levels that have been found (Alvarez-Buylla et al., 1996; Litrico et al., 2005; Jones and Hubbell, 2006; Born et al., 2008a, b, but see Davies et al., 2010). Although *C. floribundus* has a wide distribution all over disturbed areas, the effects of deforestation and habitat fragmentation may reduce genetic diversity of the populations, mainly due to the limited seed dispersal and the consequent reduction of the number of colonists and gene flow by seeds across long distances. This finding allows us to infer that other pioneer species, also with a wide distribution but dispersed over short distances or even dispersed over long distances like *Vochysia ferruginea* Mart. but with limited seed sources (Davies et al., 2010, 2013), may present similar genetic responses to forest fragmentation and fragment isolation. Thus, we suggest the creation of forest corridors and the maintenance of larger areas of forest as efficient methods for conservation of genetic resources and dynamics of pioneer trees populations. For restoration goals, we recommend collecting as many seeds as possible from many plants located at a large number of gaps (several source populations) in order to increase k and phi-values of the new established population and thus keep the genetic diversity and structure as similar as possible to a primary forest.

5. Conclusions

The genetic structure of *C. floribundus* in the primary forest was determined basically by extinctions and colonizations generated by gap dynamics and processes related to gap dynamics such as selection, inbreeding and drift. In the early successional forest, colonization seems to have stronger effects on the genetic structure of the new established populations due to the limited seed dispersal associated with the occupation of a large vacant and human-modified site. Furthermore, the effects of pollen and seed dispersal and their role on determining the number and source of colonists as well as the gene flow rates were critical to genetic diversity and structure in both forest types.

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Figure captions

Fig. 1. Map of the region of Caetetus Ecological Station, São Paulo, Brazil showing the location of the permanent plot in the primary forest and the adjacent early successional forest at Torrão de Ouro Farm.

Fig. 2. Distribution of the sampled juveniles (▲) and adults (●) of *Croton floribundus* Spreng. in the gaps of the primary forest (permanent subplots 20 x 20 m)(A) and in the sub-areas of the early successional forest (B).

Fig. 3. The relative frequency of seven cpDNA haplotypes in populations of *Croton floribundus* Spreng. in the primary forest at Caetetus Ecological Station (CES) (A) and in the early successional forest at Torrão de Ouro Farm (B). Populations of same size-class in the primary forest are represented in a unique figure.

Fig. 4. Multilocus (n=75 loci) F_{ST} estimates and 95% confidence limits for juvenile (J) and adult (A) populations of *Croton floribundus* Spreng. in the primary forest (P) at CES and in the early successional forest (E) at Torrão de Ouro Farm. Different letters indicate nonoverlapping 95% confidence intervals between groups (20 000 bootstraps).

Fig. 5. Genetic structure of *Croton floribundus* Spreng. derived from Structure analysis. Each thin vertical line represents a particular individual with their assignment proportions (y axis) to the K=3 (A) (in three different gray shades) and K=8 (B) (in eight different colours) inferred clusters. Juvenile individuals followed by adult individuals were grouped by their original gaps

and sub-areas. Labels above the figure indicate whether the populations are from the primary forest or the early successional forest. The letter codes corresponding to gaps and sub-areas (C, E, K, M) from the primary forest (P) and the early successional forest (E) are found below.

Fig. 6. SSR distogram of *Croton floribundus* Spreng. in the primary forest at CES (A). SSR distogram (B) and cpSSR correlogram (C) of *Croton floribundus* Spreng. in the early successional forest at Torrão de Ouro Farm. Spatial distance (m).

Fig. 7. SSR distograms (A-D) and cpSSR correlograms (E-F) of *Croton floribundus* Spreng. Juveniles (A) and adults (B) of *Croton floribundus* Spreng. in the primary forest at CES. Juveniles (C,E) and adults (D,F) in the early successional forest at Torrão de Ouro Farm. Spatial distance (m).

Tables

Table 1
Characteristics of the eight sampled gaps in the permanent plot at Caetetus Ecological Station (CES), four gaps for juveniles (A) and four gaps for adults (B) based on Martini et al. (2008) and AMZ Martini and RAF Lima (Unpublished results). Number of individuals sampled (N). Juvenile density of *Croton floribundus* Spreng. in each gap. Modes of tree disturbance were: UPRT - Uprooted trees; SNPT - Snapped trees; STDT - Standing trees; BRN - Branches. Gap age classes were: Age 3 (old) = tree disturbance occurred before 2002, Age 2 = tree disturbance occurred after 2002, Age 1 = tree disturbance occurred after 2002, but more recent than age 2 (for more details, see Martini et al. 2008).

(A) Juveniles

Gap	Age	Runkle area (m^2)	Mode of disturbance	Juvenile density ($ind.m^{-2}$)	N
C5	2	234	STDT, SNPT	0.15	16
E13	1	948	STDT, SNPT , BRN, UPRT	0.12	18
K8	3	291	STDT	0.85	16
M5	3	485	STDT, SNPT , BRN, UPRT	0.13	14

(B) Adults

Gap	Age	Runkle area (m^2)	Mode of disturbance	Adult density ($ind.m^{-2}$)	N
B6[a]	>3	270	UPRT, STDT	-	11
D12	3	657	STDT, SNPT	-	11
L7	3	488	SNPT	-	13
M6[a]	>3	393	STDT, SNPT,	-	14

[a] Gaps characterized by the authors following the method of Lima et al. (2008).

Table 2 Genetic diversity within populations of N individuals of *Croton floribundus* Spreng. in the primary forest at Caetetus Ecological Station (CES) and in the early successional forest at Torrão de Ouro Farm assessed by average number of alleles per locus (*A*), effective number of alleles per locus (A_e), allelic richness (*R*), number of private alleles (A_p), ratio of private alleles over average number of alleles per locus (A_p/A), proportion of polymorphic loci (*P'*), gene diversity averaged over all loci ($\underline{H}$ and $\underline{H}'$), and Shannon's genetic index averaged over all loci ($\underline{H}'_{Shannon}$).

			N	*A*	A_e	*R*	A_p	A_p/A	$\underline{H}$	*P'* (%)	$\underline{H}'$	$\underline{H}'_{Shannon}$
Primary Forest	**Juveniles**	**C**	16	7.6	5.0	7.11	1	0.13	0.745	61	0.195	0.408
		E	18	7.7	5.1	7.11	2	0.26	0.770	65	0.210	0.436
		K	16	7.7	4.8	7.07	3	0.34	0.760	62	0.191	0.403
		M	14	6.4	5.0	7.11	0	0	0.797	52	0.198	0.398
		Mean		7.4	5.0	7.10	1.5	0.20	0.768	60	0.198	0.411
	Adults	**C**	11	5.7	4.4	7.00	0	0	0.769	44	0.160	0.318
		E	11	6.9	5.3	7.14	0	0	0.814	53	0.209	0.407
		K	13	6.9	4.8	7.08	1	0.15	0.787	53	0.186	0.376
		M	14	7.0	5.2	7.13	0	0	0.793	57	0.207	0.417
		Mean		6.6	4.9	7.09	0.3	0.04	0.791	52	0.190	0.380
Early Successional Forest	**Juveniles**	**C'**	16	7.1	5.0	7.09	1	0.14	0.784	56	0.187	0.386
		E'	18	7.3	4.9	7.06	0	0	0.720	60	0.192	0.403
		K'	14	6.4	4.7	7.05	0	0	0.696	48	0.168	0.344
		M'	16	7.0	4.9	7.05	1	0.14	0.749	57	0.184	0.384
		Mean		7.0	4.8	7.06	0.5	0.07	0.737	55	0.183	0.379
	Adults	**C'**	11	5.9	4.6	7.02	0	0	0.785	38	0.115	0.240
		E'	11	6.9	4.9	7.11	0	0	0.798	56	0.196	0.394
		K'	13	5.7	4.0	6.90	1	0.18	0.674	38	0.089	0.200
		M'	13	6.0	4.9	7.06	0	0	0.791	45	0.181	0.357
		Mean		6.1	4.6	7.02	0.3	0.04	0.762	44	0.145	0.298

Table 3
Number of haplotypes (n), haplotype richness (r) number of private haplotypes (n_p), and haplotype diversity (h) in 16 populations of *Croton floribundus* Spreng. in the primary forest at Caetetus Ecological Station (CES) and in the early successional forest at Torrão de Ouro Farm.

			n	r	n_p	h
Primary Forest	**Juveniles**	**C**	1	0	0	0
		E	1	0	0	0
		K	1	0	0	0
		M	1	0	0	0
	Adults	**C**	1	0	0	0
		E	1	0	0	0
		K	1	0	0	0
		M	1	0	0	0
Early Successional Forest	**Juveniles**	**C'**	3	1.3	1	0.342
		E'	5	3.0	0	0.787
		K'	4	2.6	0	0.712
		M'	3	2.0	0	0.718
	Adults	**C'**	1	0	0	0
		E'	2	0.7	0	0.182
		K'	2	1.0	0	0.250
		M'	2	1.0	0	0.509

Table 4

Results of Hierarchical Analysis of Molecular Variance (AMOVA) for different groups of populations of *Croton floribundus* Spreng. using nuclear and chloroplast SSR markers in the primary forest at Caetetus Ecological Station (CES) and in the early successional forest at Torrão de Ouro Farm. Fixation indices could not be calculated for populations of the primary forest using cpSSR markers. Estimates statistically different from zero ($p < 0.05$) are in bold. F_{SC} indicates the differentiation among populations within groups. F_{CT} indicates the differentiation among groups. F_{ST} indicates the differentiation among populations among groups or only the differentiation among populations.

		Nuclear SSR			**cpSSR**		
	Groups	F_{SC}	F_{ST}	F_{CT}	F_{SC}	F_{ST}	F_{CT}
Primary Forest	Size classes	**0.105*** **	**0.108*** **	0.003	-	-	-
	Juveniles	-	**0.081*** **	-	-	-	-
	Adults	-	**0.139*** **	-	-	-	-
	Gaps	**0.083*** **	**0.110*** **	**0.029***			
	All populations	-	**0.107*** **	-	-	-	-
Early Successional Forest	Size classes	**0.254*** **	**0.212*** **	-0.057	**0.439*** **	**0.538*** **	0.177
	Juveniles	-	**0.165*** **	-	-	**0.328*** **	-
	Adults	-	**0.370*** **	-	-	**0.653*** **	-
	Sub-areas	**0.060*** **	**0.253*** **	**0.206*** **	**0.363*** **	**0.516*** **	0.240
	All populations	-	**0.231*** **	-	-	**0.499*** **	-
All populations	Size classes	**0.196*** **	**0.183*** **	-0.016	**0.683*** **	**0.683*** **	0.000
	Forest types (juveniles)	**0.123*** **	**0.157*** **	**0.038*** **	**0.330*** **	**0.623*** **	**0.437***
	Forest types (adults)	**0.258*** **	**0.274*** **	0.022	**0.655*** **	**0.898*** **	**0.704***
	Gaps and sub-areas	**0.072*** **	**0.196*** **	**0.133*** **	**0.367*** **	**0.694*** **	**0.517*** **
	Forest types	**0.169*** **	**0.206*** **	**0.045*** **	**0.501*** **	**0.760*** **	**0.519****

* $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

FIGURES

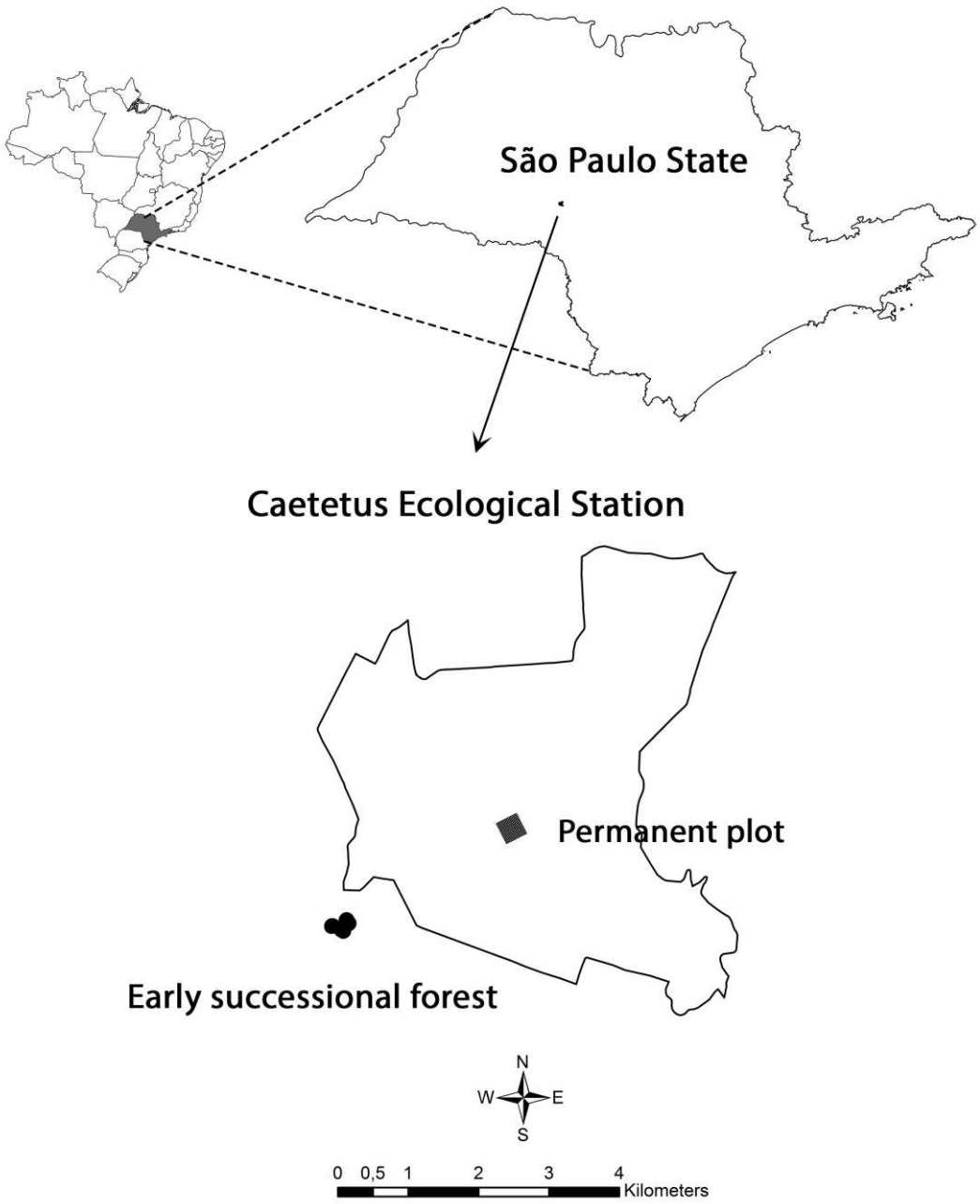
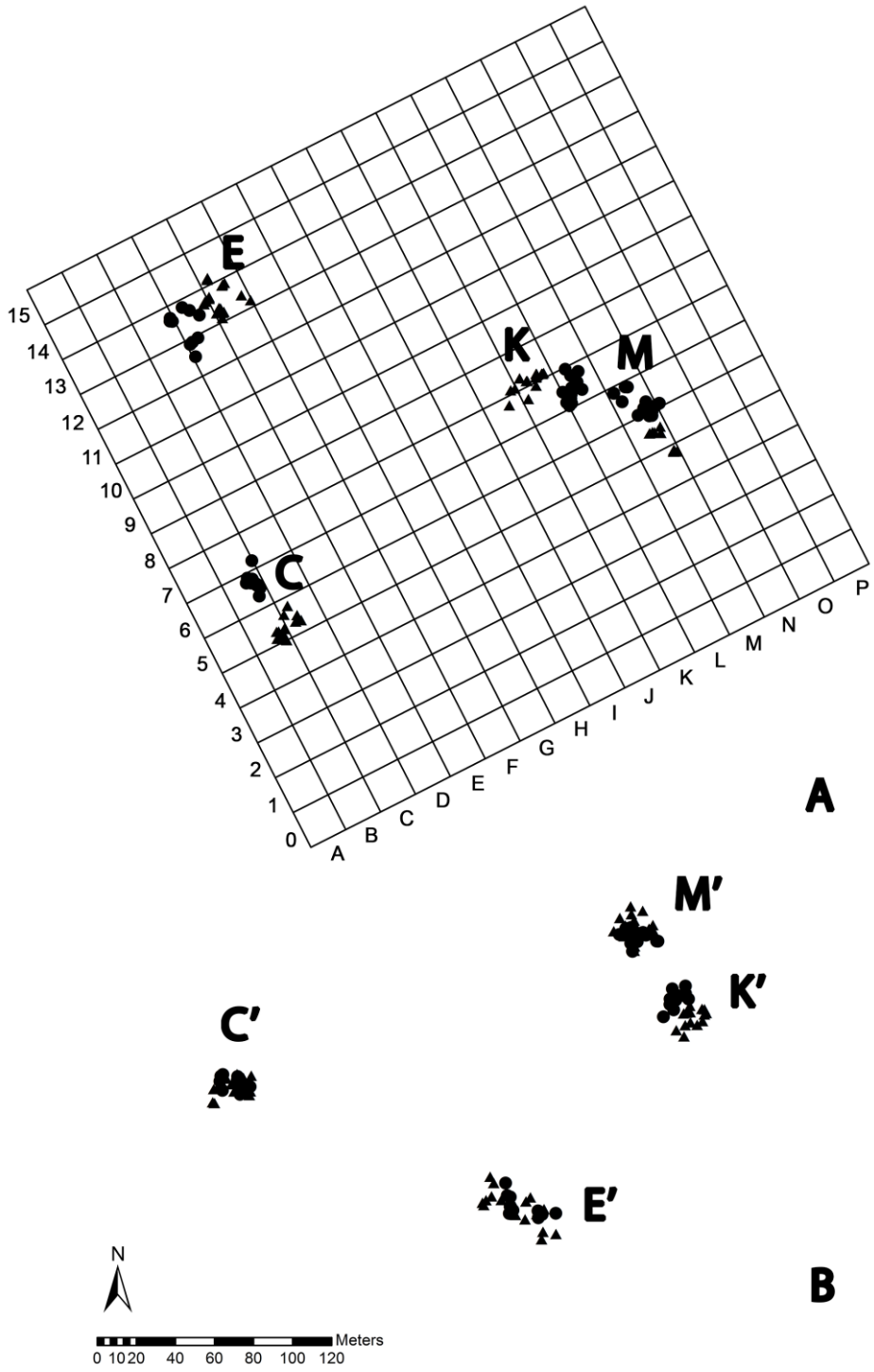


Figure 1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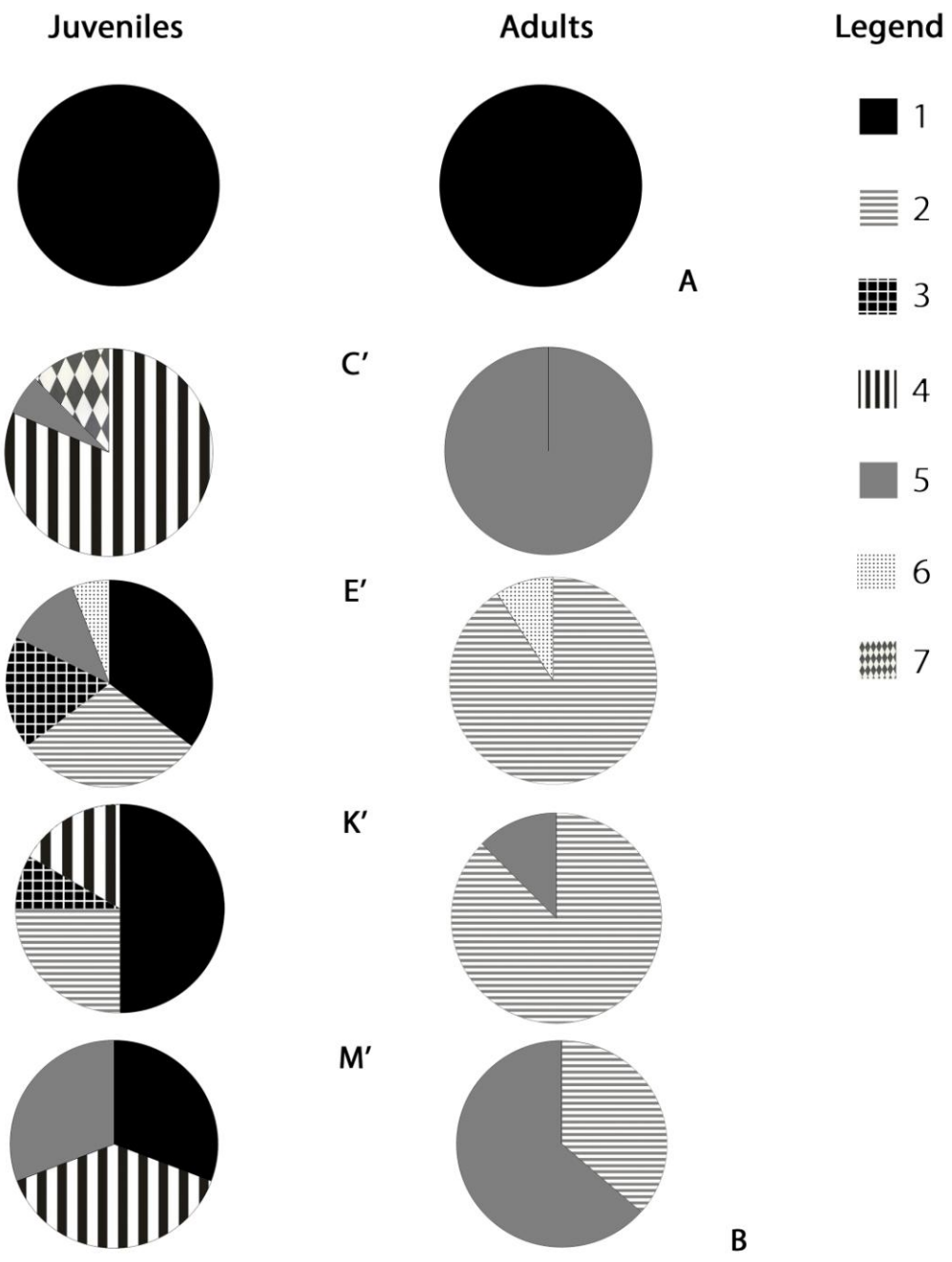

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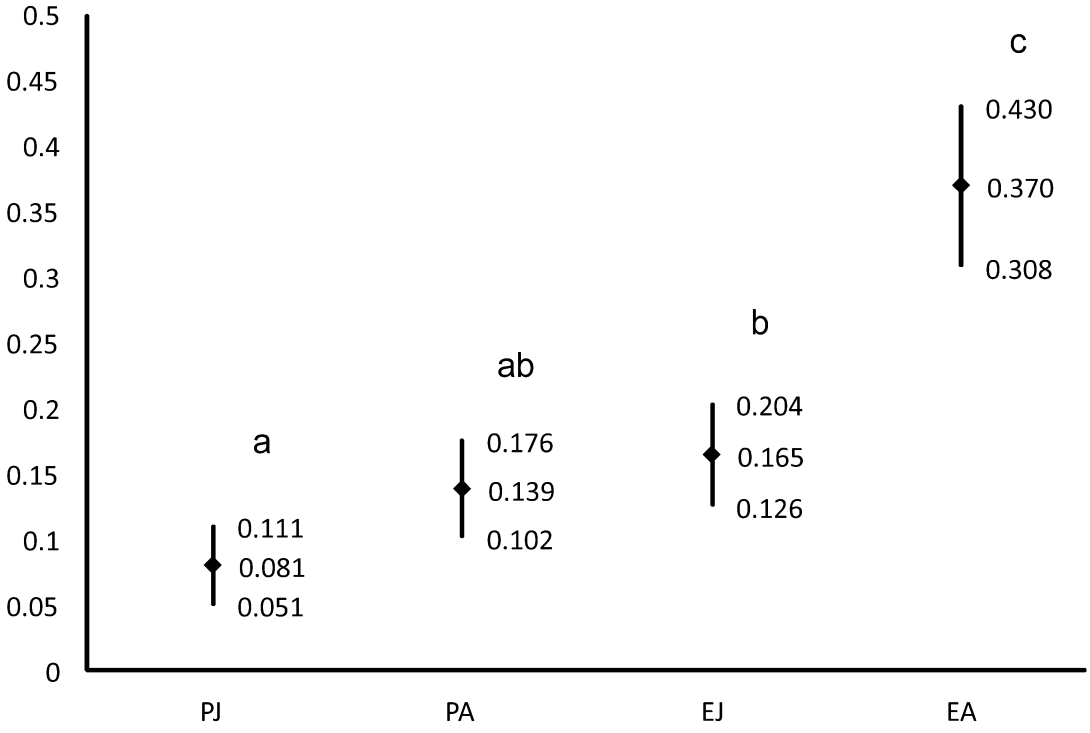
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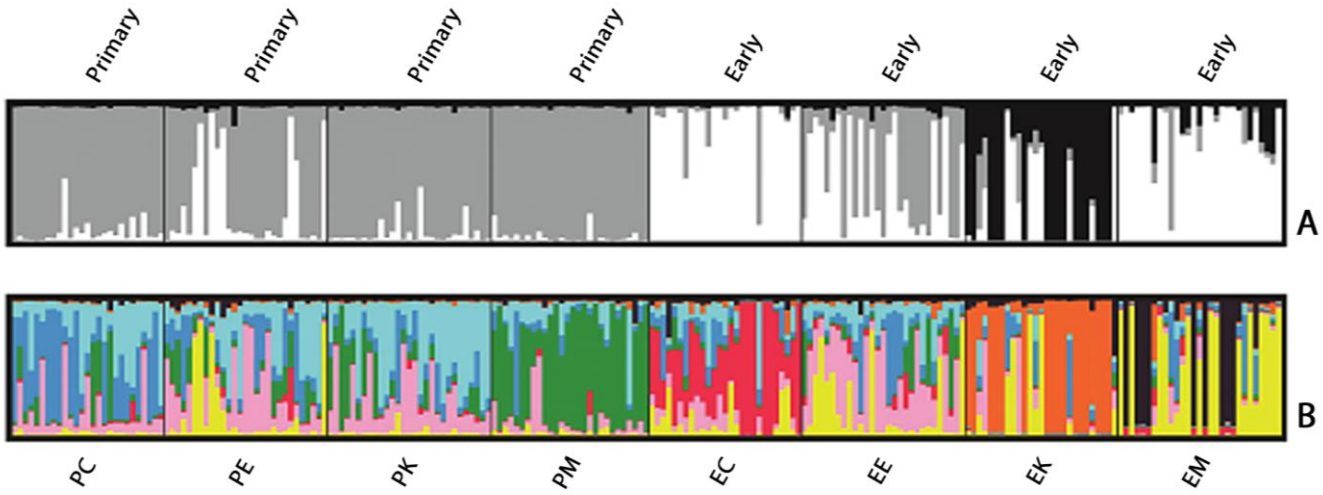
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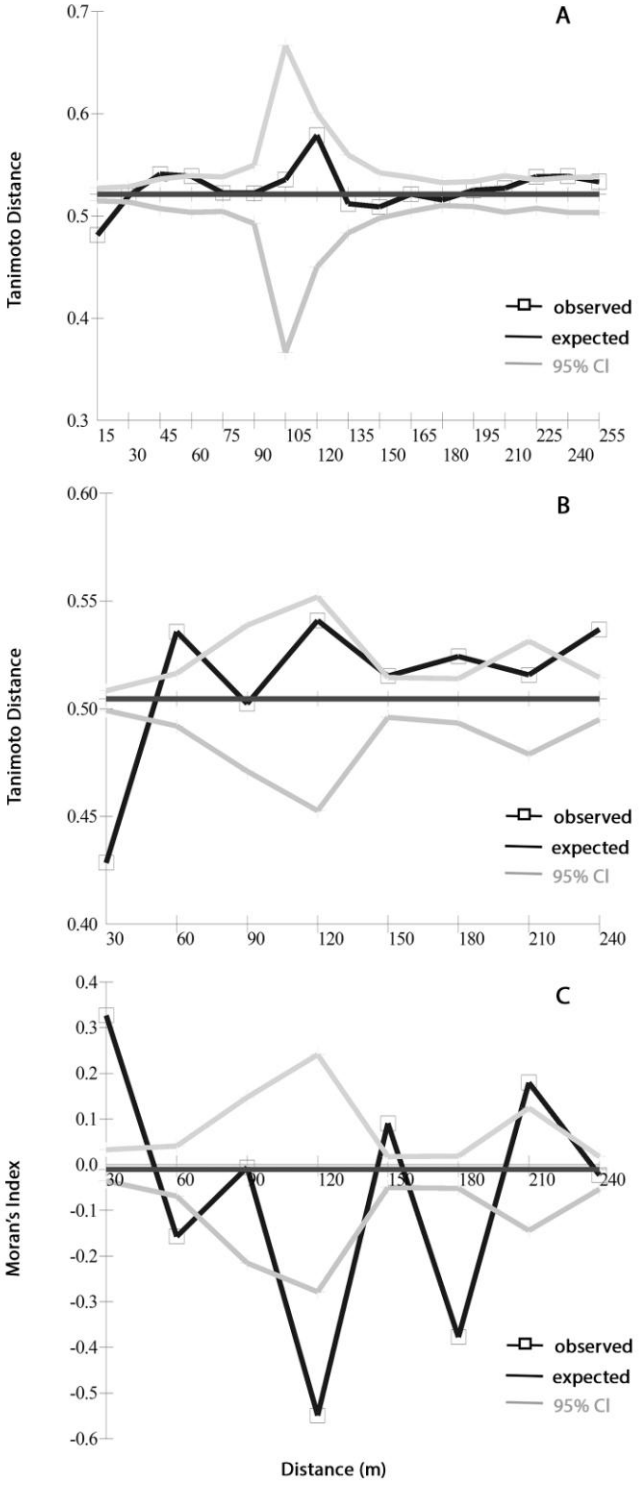
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Figure 6

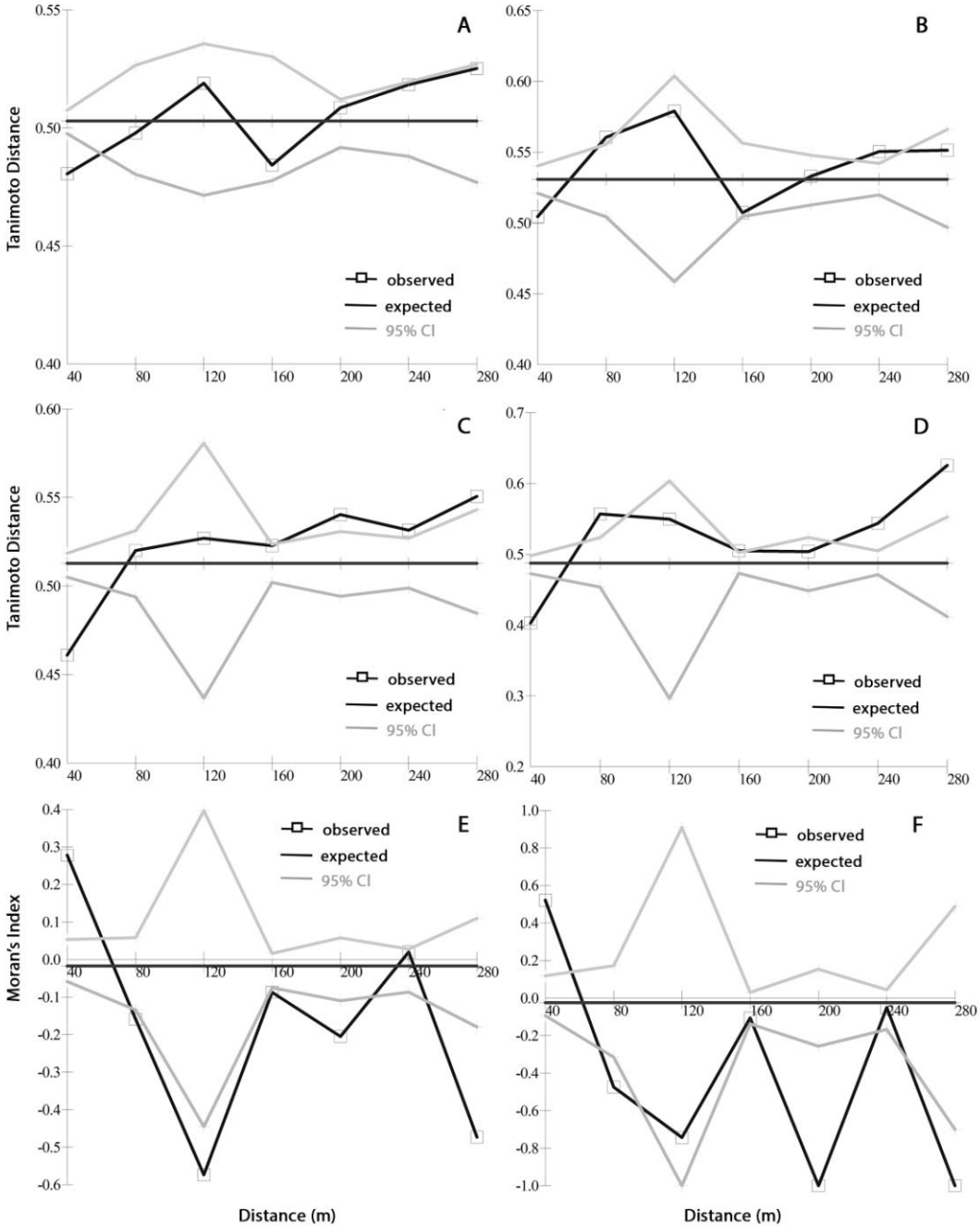
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Figure 7

Supplementary material

Table A1
Particle-size distribution ($g.Kg^{-1}$) of the different soil horizon layers sampled at sub-areas C', E', K' and M' in the early successional forest at Torrão de Ouro Farm. Two sites were sampled at the E' sub-area: E'_W and E'_E.

		Soil horizons/depths	Sand (50-2000μm)	Silt (2-50μm)	Clay (<2μm)
C'		0-0.05m AE	869	30	101
		0.05-0.40m AE	906	19	75
		0.40-0.60m E	889	111	75
		0.60-1.20m +Bt	635	32	332
E'	W	0-0.45m A	781	41	178
		0.60-0.80m E	894	31	75
		0.80-1.10m C	867	33	100
	E	0-0.35m A	827	45	129
		0.35-0.70m E	815	12	174
		0.70-1.20m Btg	701	23	276
K'		0-0.10m A	891	33	76
		0.10-0.30m AE	902	22	75
		0.30-0.70m E	917	33	50
		0.70-1.20m +Bt	694	50	256
M'		0-0.10m AE	849	24	126
		0.10-0.30m AE	834	17	148
		0.30-0.75m E	881	20	99
		0.75-1.20m +Bt	698	46	256

Table A2 Chemical properties of the different soil horizon layers sampled at sub-areas C', E', K' and M' in the early successional forest at Torrão de Ouro Farm. Two sites were sampled at the E' sub-area: E'_W and E'_E.

	Soil horizons/depths	pH_{H2O}	pH_{KCl}	Organic Materials	P	Exchangeable bases					H+Al	Cation Exchange Capacity	Base Saturation	Aluminum saturation
						Na	K	Ca	Mg	Al				
				$g.Kg^{-1}$	$mg.Kg^{-1}$	$mmol_c.\ Kg^{-1}$							%	
C'	0-0.05m AE	6.0	4.7	11	1	0.0	2.7	17	6	1	9	34.4	75	4
	0.05-0.40m AE	5.9	4.6	10	1	0.0	1.1	10	2	2	4	16.6	76	11
	0.40-0.60m E	5.8	4.3	8	1	0.0	0.4	9	2	1	4	14.7	72	12
	0.60-1.20m +Bt	5.6	3.9	26	1	0.1	1.4	42	20	14	15	79.2	81	18
E' W	0-0.45m A	5.1	3.8	6	2	0.1	2.4	35	4	10	26	67.9	61	20
	0.60-0.80m E	5.6	4.4	11	1	0.1	0.4	16	1	1	4	21.0	79	7
	0.80-1.10m C	5.9	4.5	21	1	0.1	0.5	28	1	1	3	33.2	90	2
E	0-0.35m A	5.2	3.9	9	3	0.1	1.4	28	4	5	17	50.7	66	14
	0.35-0.70m E	5.7	4.1	5	1	0.1	0.8	41	7	2	6	54.6	89	5
	0.70-1.20m Btg	6.3	4.7	12	1	0.2	2.0	83	22	1	4	111.3	96	1
K'	0-0.10m A	6.9	6.3	23	2	0.1	3.8	54	19	0	1	78.4	99	0
	0.10-0.30m AE	6.9	5.6	8	1	0.1	2.9	14	11	0	3	31.5	92	0
	0.30-0.70m E	6.7	5.4	4	1	0.1	2.5	10	2	0	2	16.6	89	0
	0.70-1.20m +Bt	5.9	4.2	8	1	0.2	2.3	64	19	4	10	94.6	90	4
M'	0-0.10m AE	4.5	5.6	17	1	0.1	3.0	53	16	0	5	77.0	93	0
	0.10-0.30m AE	5.9	4.8	18	1	0.0	2.2	30	11	1	12	55.1	78	1
	0.30-0.75m E	5.9	4.6	9	1	0.0	1.0	17	2	1	5	24.6	81	5
	0.75-1.20m +Bt	5.7	4.0	8	1	0.2	2.7	72	20	4	11	104.9	90	4

Table A3
Characteristics of seven microsatellite loci amplified in *Croton floribundus* Spreng. in the primary forest at Caetetus Ecological Station (CES) and in the early successional forest at Torrão de Ouro Farm. Forward (F) and reverse (R) primer sequences, repeat motif, product size range in base pairs, number of alleles per locus (N_a), and number of alleles per individuals in each locus (N_i).

Locus code	Sequence of primers (5 '- 3')	Repeat motif	Size range	N_a	N_i
SSR-5	F= CGTGTGCTTCCTCTTCACC R= GCCAAATGGGTTTCTCGTTA	$(AAG)_8N_{18}(CT)_4$	142-183	13	3-7
SSR-6	F= CCACATTCCACATCACTTCC R= ACGTGGCAAAATGGGTAGTC	$(GA)_5$	203-213	6	1-3
SSR-8	F= CTCCTGAACCCACGCTAGAT R= AGAGGCTTCGTGTTGTTCGT	$(AG)_8$	214-216	2	1-2
SSR-10	F= AGACGGAAGGGAAGTGGAGT R= CGCAGCATCATATCACCAGA	$(AAG)_8$	141-187	14	3-8
SSR-12	F= TTGGGCAGATTCATGTAACG R=TTGGTTGAGGGAACAGATGA	$(GAA)_8N_{37}(AG)_3$	157-202	19	1-7
SSR-14	F= AGCACTCCTTCCACGACATT R=TGGTGAGACGCTTCATGTTG	$(CA)_{11}$	192-211	8	1-2
SSR-16	F= GGCAGGCATAAATCAGCAAT R=TATCGAATCGTCGGATTTGG	$(GAA)_6$	200-255	15	2-8

Table A4
Locus code, foward (F) and reverse (R) primer sequences, repeat motif, number of alleles per locus (N_a), and product size range in base pairs (bp) of the six universal choroplast microsatellite loci amplified in *Croton floribundus* Spreng. in the primary forest at Caetetus Ecological Station (CES) and in the early successional forest at Torrão de Ouro Farm.

Locus code	Sequence of primers (5 '- 3')[a]	Repeat Motif[a]	N_a	Size range
ccmp 2	F= ATCCCGGACGTAATCCTG R= ATCGTACCGAGGGTTCGAAT	$(A)_{11}$	2	301-311
ccmp 3	F= CAGACCAAAAGCTGACATAG R= GTTTCATTCGGCTCCTTTAT	$(T)_{11}$	2	104-110
ccmp 4	F= AATGCTGAATCGAYGACCTA R= CCAAAATATTBGGAGGACTCT	$(T)_{13}$	2	112-113
ccmp 5	F= TGTTCCAATATCTTCTTGTCATTT R= AGGTTCCATCGGAACAATTAT	$(C)_7(T)_{10}$ $(T)_5C(A)_{11}$	1	109
ccmp 7	F= CAACATATACCACTGTCAAG R= ACATCATTATTGTATACTCTTTC	$(A)_{13}$	1	130
ccmp 10	F= TTTTTTTTTAGTGAACGTGTCA R= TTCGTCGDCGTAGTAAATAG	$(T)_{14}$	1	134

[a] According to Weising and Gardner (1999).

Table A5

Linkage disequilibrium values (D), standardized linkage disequilibrium values (D'), and associated probabilities estimated for pairs of the commonest nuclear alleles and chloroplast haplotypes of *Croton floribundus* Spreng. in the early successional forest at Torrão de Ouro Farm. D' values statistically different from zero according to G-tests of independence (α =0.05) are followed by *. D' values statistically different from zero according to Bonferroni method (α =0.0015) are in bold.

Nuclear allele/cpSSR haplotype pair	D	D'	p-value
SSR-5_151/1	0.002	1	0.6590
SSR-5_167/1	-0.004	-0.048	0.8252
SSR-5_173/1	-0.011	-0.134	0.5550
SSR-6_205/1*	-0.038	-1*	0.0163
SSR-8_216/1	0.022	0.524	0.1719
SSR-10_157/1	-0.009	0.083	0.4674
SSR-12_174/1	0.017	0.462	0.2669
SSR-14_192/1	-0.007	-0.147	0.6803
SSR-16_211/1	-0.028	-0.314	0.1318
SSR-16_214/1	0.028	0.578	0.1015
SSR-16_223/1	-0.011	0.079	0.4681
SSR-5_151/2	-0.007	-0.999	0.5177
SSR-5_167/2	-0.033	0.212	0.1529
SSR-5_173/2*	0.064	0.443*	0.005
SSR-6_205/2	-0.068	**-1**	**0.0004**
SSR-8_216/2	0.037	0.475	0.0696
SSR-10_157/2*	-0.042	-0.456*	0.0061
SSR-12_174/2	0.028	0.406	0.1523
SSR-14_192/2	0.025	0.120	0.2242
SSR-16_211/2	-0.040	-0.243	0.0867
SSR-16_214/2	0.016	0.184	0.4432
SSR-16_223/2	0.025	0.443	0.1793
SSR-5_151/5	-0.007	-1	0.0922
SSR-5_167/5	-0.007	-0.048	0.7640
SSR-5_173/5	-0.032	-0.238	0.1526
SSR-6_205/5*	0.050	0.292*	0.0073
SSR-8_216/5	-0.032	-0.165	0.0997
SSR-10_157/5	0.024	0.707	0.1026
SSR-12_174/5	-0.030	-0.175	0.1095
SSR-14_192/5	-0.006	-0.081	0.7572
SSR-16_211/5	0.091	**0.746**	**<0.0001**
SSR-16_214/5*	-0.044	-0.235*	0.0309
SSR-16_223/5	0.009	0.171	0.6267

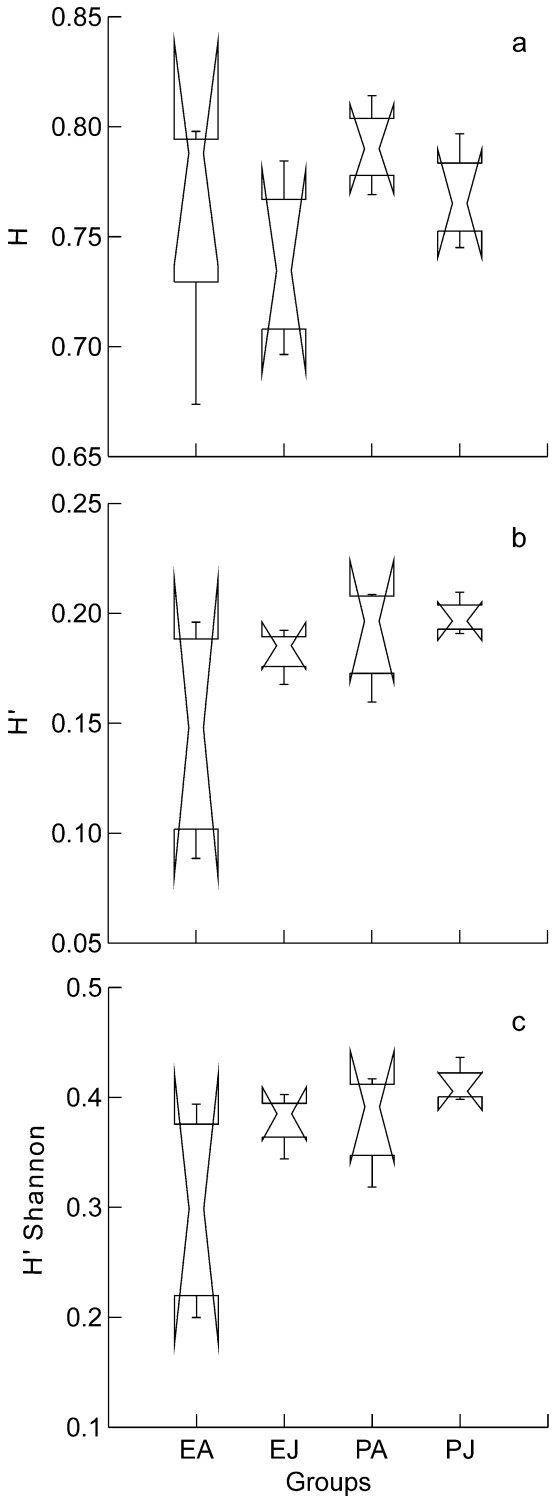

Fig. A1. Notched box plots of genetic diversity indices: H (a), H' (b), and $H'_{Shannon}$ (c) in populations of adults (A) and juveniles (J) of *Croton floribundus* Spreng. in the primary forest (P) at Caetetus Ecological Station and in the early successional forest (E) at Torrão de Ouro Farm. Overlapping of median confidence intervals indicates similarity of groups with 95% confidence level.

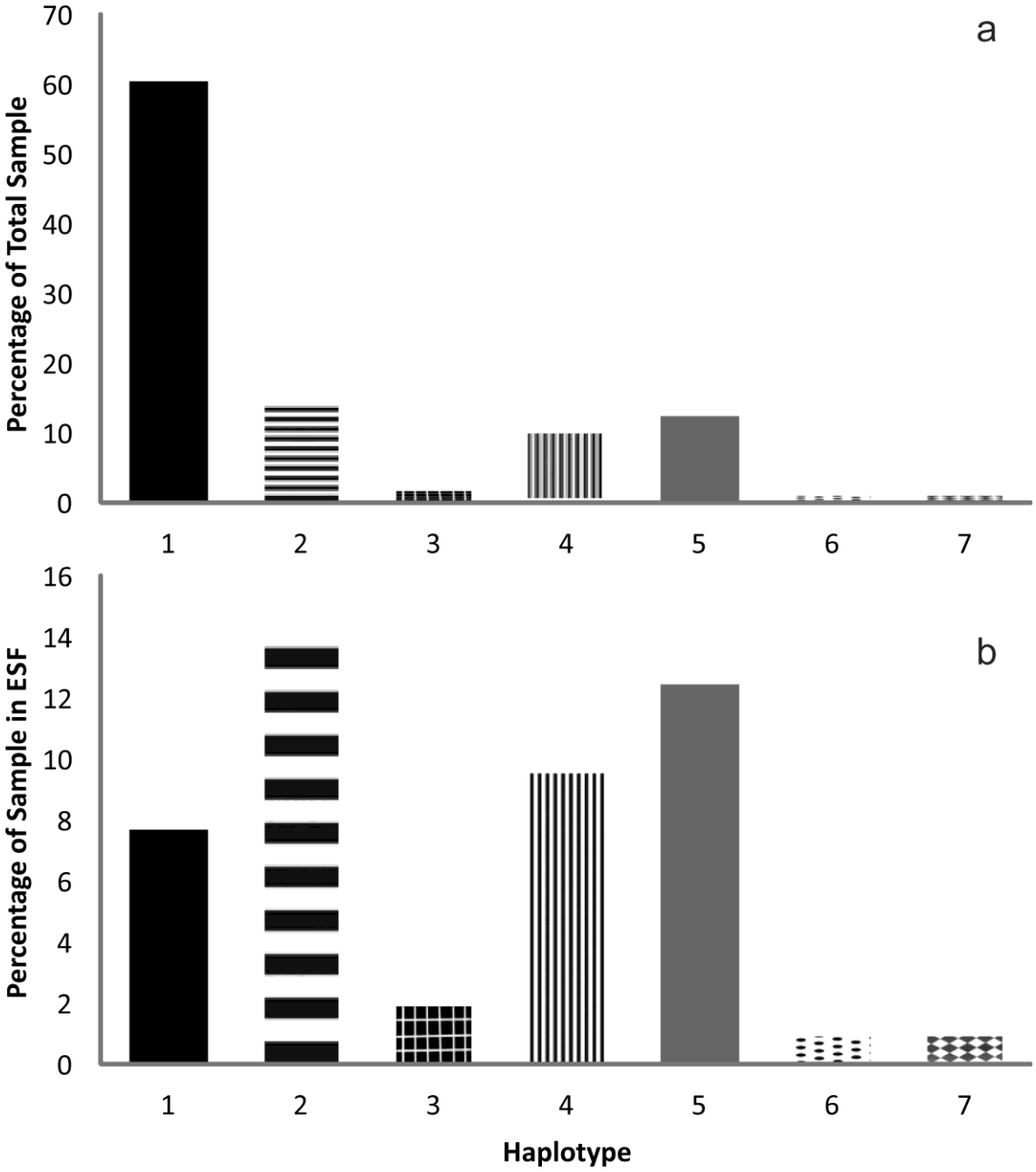

Fig. A2. The relative frequency of seven cpSSR haplotypes after pooling all individuals of *Croton floribundus* Spreng. sampled from both study sites (a) and after pooling all individuals sampled from early successional forest (ESF)(b).

CONCLUSIONS

This study showed that the population genetics of the pioneer species *C. floribundus* is closely related to its ecology and reproductive biology. Not only did the population structure of the species vary according to the forest type, but also the distribution of genetic diversity among populations. The following ecological, genetic and cytogenetic characteristics of the species were found:

- *C. floribundus,* a long lived pioneer tree species with rapid growth, was widespread and equally distributed along the gap range, but its population structure varied between areas with contrasting levels of human disturbance. The results indicate that the high intensity and different types of disturbance produced by human land use can alter the underlying conditions under which the main ecological processes responsible for plant distribution in tropical forests act.

- The six universal chloroplast microsatellite loci optimized for *C. floribundus* were useful for comparisons with nuclear markers and the subsequent inferences about colonization process and seed and pollen dispersal in the species.

- The seven nuclear microsatellite developed and optimized for *C. floribundus* showed to be efficient tools to evaluate the patterns of genetic diversity and structure in the species.

- Chromosome number counted from pollen mother cells showed a number of bivalents n=56 (2n=8x=112) in all populations studied of *C. floribundus*, i.e.,

regions of Caetetus Ecological Station, Gália/Alvinlândia, SP; Santa Genebra Reserve, Campinas, SP; and Ribeirão Cachoeira, Sousas, Campinas, SP.

- The normal meiotic pairing and the high pollen viability suggested that *C. floribundus* is a regular and stable polyploid, much likely an allopolyploid.

Based on these features and the SSR and cpSSR markers optimized for the species, we were able to answer the major question of the study:

Are the genetic diversity and fine-scale structure of the pioneer tree *Croton floribundus* Spreng. (Euphorbiaceae) affected by the specific ecological and genetic factors of the primary and early successional forests generated by disturbances?

The main findings were:

- High genetic diversity levels for *C. floribundus* in the primary and the early successional forests studied;

- Origin of populations, allelic richness, and genetic structure of *C. floribundus* differed between the primary and the early successional forests studied;

- Combined effects of seed dispersal and colonization (and extinctions) were determinants of the fine-scale genetic structure of *C. floribundus* in both forest types:

    a) In the primary forest, processes related to gap dynamics such as differential survival of genotypes, inbreeding and genetic drift also affected genetic structure by increasing F_{ST}, especially for adults;

b) In the early successional forest, human disturbances boosted the founder effects in *C. floribundus* by increasing genetic differentiation among the new established populations much likely due to its limited seed dispersal associated with the occupation of a large vacant and ecologically modified site.

- Gene flow by pollen was important for maintaining the genetic diversity within populations of *C. floribundus* in both primary and early successional forests. In the early successional forests, gene flow by seeds seemed to be also determinant.

The results contributed to understanding the changes in genetic diversity and structure of *C. floribundus* across successive size classes in sites with contrasting levels of human disturbance and the determinant factors of these changes.

We observed that although *C. floribundus* has a wide distribution all over disturbed areas, the effects of deforestation and habitat fragmentation may reduce genetic diversity of the populations, mainly due to the limited seed dispersal and the consequent reduction of colonists and gene flow by seeds across long distances. This finding allows us to infer that other pioneer species, also with a wide distribution but dispersed over short distances or with limited seed sources, may present similar genetic responses to forest fragmentation and fragment isolation. To our knowledge, this is the first attempt to isolate the ecological factor level of human disturbance in a study of population genetics of a pioneer tropical tree species. Because this thesis showed that the gap dynamics and colonization of

human disturbed areas affect the genetic diversity and structure of pioneer species, we believe that future genetic studies of these species must take in account their distribution in gaps as well as in secondary forests with different levels and types of human disturbances for the establishment of appropriate study designs. Also, the increase in knowledge of such aspects of the pioneer species is essential for understanding the effects of human disturbance and of successional processes in the tropical forests, which in turn is important for forest conservation, management and restoration.

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