FEDERAL UNIVERSITY OF PARANA

LUCIANO MEDINA MACEDO

GENETIC VARIABILITY OF *Araucaria angustifolia* IN SOUTHERN BRAZIL TO EVALUATION OF ENVIRONMENTAL SERVICES IN ARAUCARIA FOREST AREAS UNDER DIFFERENT HISTORICAL USE

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Thesis presented as partial requirement for obtaining the Doctor degree in Bioprocess Engineering and Biotechnology, Agroindustry Area. Post-graduation Program in Bioprocess Engineering and Biotechnology, Federal University of Parana.

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RELATÓRIO DE DEFESA DE TESE DE DOUTORADO

Aos dezenove dias do mês de agosto 2014, na Sala de Aula do CENBAPAR do Centro Politécnico da Universidade Federal do Paraná, Jardim das Américas, foi instalada pela Profª Drª Luciana Porto de Souza Vandenberghe, Coordenadora do Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, a banca examinadora para a Nonagésima Nona Defesa de Tese de Doutorado, Área de Concentração: Agroindústria e Biocombustíveis. Estiveram presentes no Ato, além da Coordenadora do Curso de Pós–Graduação, professores, alunos e visitantes.

A Banca Examinadora, atendendo determinação do colegiado do Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, ficou constituída pelos Professores Doutores André Luis Lopes da Silva (UFPR), André Eduardo Biscaia de Lacerda (EMBRAPA), Juliana Degenhardt Goldbach (EMBRAPA), Juliana Vitória Messias Bittencourt (UTFPR) e Carlos Ricardo Soccol (UFPR– orientador da tese).

Às 14h00, a banca iniciou os trabalhos, convidando o candidato **Luciano Medina Macedo** a fazer a apresentação da Tese intitulada: **"**GENETIC VARIABILITY OF *Araucaria angustifolia* IN SOUTHERN BRAZIL TO EVALUATION OF ENVIRONMENTAL SERVICES IN ARAUCARIA FOREST AREAS UNDER DIFFERENT HISTORICAL USE**"**. Encerrada a apresentação, iniciou-se a fase de argüição pelos membros participantes.

Tendo em vista a tese e a argüição, a banca composta pelos professores Dr André Luis Lopes da Silva, Dr André Eduardo Biscaia de Lacerda, Drª Juliana Degenhardt Goldbach, Drª Juliana Vitória Messias Bittencourt e Dr Carlos Ricardo Soccol declarou o candidato Aprovado (de acordo com a determinação dos Artigos 59 a 68 da resolução 65/09 de 30.10.09).

Curitiba, 19 de agosto de 2014

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RESUMO

A avaliação dos serviços ambientais em florestas nativas é uma abordagem conservacionista recente, que tem como objetivo incentivar a conservação de áreas com bioma nativos em propriedades rurais particulares. Áreas relavantes para determinados biomas podem ser priorizadas para receberem compensações por seus ativos ambientais, seja na forma de pagamentos em espécie ou incentivos fiscais aos seus proprietários. Entretanto, uma das maiores dificuldades está na definição dos critérios de elegibilidade para identificar áreas relevantes, pois estas precisam ser selecionadas de acordo com o bioma e a finalidade do programa de serviço ambiental. Esta tese teve como objetivo avaliar parâmetros genéticos da espécie *Araucaria angustifolia* em diferentes cenários, em busca de parâmetros científicos que possam ser utilizados em estratégias de conservação do bioma da Floresta com Araucária. Historicamente a Floresta com Araucária proveu madeira e vida selvagem em abundância para os primeiros colonizadores, mas o desenvolvimento trouxe como consequência a redução das áreas em estágios primários de conservação para menos de 5% de sua área original. Atualmente extensas áreas de Floresta com Araucária são raras e muitos remanescentes são pequenos e fisicamente desconectados na paisagem. Foram coletadas sementes de *A. angustifolia* em quatro locais com diferentes históricos de conservação e uso da terra aos redores da Estação Experimental da EMBRAPA em Caçador - SC, um dos maiores maciços contínuos da Floresta com Araucária. Adicionalmente, foi realizado um senso nas árvores de Araucária adultas em um fragmento florestal de sete hectares (295 árvores). A coleta e isolamento de DNA destas árvores adultas exigiu o ajuste de uma nova metodologia, que apresenta maior produtividade na etapa de campo e pode ser aplicada em uma grande variedade de espécies florestais de grande porte. A diversidade genéticas das amostras foram avaliadas utilizando marcadores microssatélites (10 *loci*) em três sistemas multiplex. As análises de paternidade indicaram dentro de cenários florestais a elevada densidade de doadores de pólen compensa o menor fluxo de vento e pólen. Adicionalmente, foi comprovado que quanto maior a extensão e o estado de conservação da floresta, maior será a diversidade existente. Analisando a diversidade de sementes coletadas em cenários contrastantes, verificou-se que remanescentes florestais fisicamente isolados até 2 km de distância de florestas contínuas são capazes de estabelecer conectividade funcional através do fluxo gênico, produzindo sementes com elevada variabilidade e reduzida endogamia. Embora remanescentes situados a 5 km de distância ou mais de contínuos florestais não sejam capazes de estabelecer conectividade funcional devido seu isolamento pela distância, estas áreas podem apresentar novos níveis de diversidade (alelos), não presentes na floresta contínua. Os resultados também demonstraram que para reter um montante de 150 indivíduos não relacionados, é necessário coletar sementes de 45 árvores localizadas próximas a cenários florestais, ou de 56 árvores em condições isoladas. Concluindo, os parâmetros genéticos indicam que áreas não distantes mais que 2 km de grandes maciços florestais devem ser priorizadas para receberem serviços ambientais no bioma da Floresta com Araucária, e as amostragens de sementes para fins conservação da espécie *A. angustifolia* devem ser realizadas quando possível em locais distantes mais de 5 km entre si.

Palavras-chave: Araucária, diversidade, marcadores microssatélites, serviços ambientais.

ABSTRACT

The evaluation of ecosystem services in natural forests is a conservationist approach that aims to motivate the preservation of natural biomes in private rural areas. Areas that are important for ecosystem services in identified biomes can be prioritized in programs through monetary payments or the establishment of fiscal incentives for landowners. However, one of the major hurdles in applying this approach is defining the criteria used to establish relevant areas, because protected areas need to be defined based on the biome and the scope of each ecosystem service program. This thesis seeks to evaluate the genetic diversity of the *Araucaria angustifolia* species in different scenarios, to define scientific parameters to be used in conservation strategies of the Araucaria Forest biome. Traditionally, the Araucaria Forest provided wood and wildlife for colonizers; however, economic development has led to the reduction of pristine Araucaria Forest areas to less than 5% of the original forest area. Today, extensive areas with Araucaria Forest are rare and many of the remnants are small and physically isolated. *A. angustifolia* seeds were collected in four sites with different conservation and land use history in and around the EMBRAPA Research Station in Caçador, Santa Catarina State, one of largest continuous areas with Araucaria Forest in Brazil. Additionally, a census of *A. angustifolia* adult trees was conducted in a seven hectare forest remnant (295 trees sampled). The collection of samples from adult trees for DNA isolation required the development of a new methodology which is more efficient during fieldwork and can be applied to a wide range of tall tree species. Genetic diversity was assessed using microsatellite markers (10 loci) in three multiplex systems. The paternity analysis showed a high number of pollen donors, despite different types of land use. This result is a reflection of extensive pollen dispersion by wind. Additionally, the results demonstrate that larger forested areas with greater forest conservation result in higher levels of genetic diversity. The results for genetic diversity in seeds comparing different types of land use show that physically isolated forest remnants located 2 km from a continuous forest are able to maintain functional connectivity through gene flow, producing seeds with high levels of diversity and low endogamy. Remnants located 5 km from the continuous forest are not able to establish functional connectivity due to isolation by distance; however, these areas contain diversity (alleles) that are not present in the continuous forest. The results also show that to retain an effective population size of 150 unrelated individuals, it is necessary to collect seeds from 45 trees located near forest scenarios, or 56 trees located in isolated conditions. Finally, the results of the genetics analyses suggest that fragments located no more than 2 km from large continuous forests should be prioritized for ecosystem services programs in the Araucaria Forest biome. Seed sampling strategies for the conservation of *A. angustifolia* species should be realized on a landscape scale with a distance of at least 5 km between sampling sites.

Key-words: Araucaria, diversity, microsatellites markers, ecosystem services.

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LIST OF ACRONYMS

CNPq	-	Brazilian Agency for Scientific and Technological Development
CAPES	-	Coordination for the Improvement of Post-Graduate Education
CIA	-	Chloroform Isoamyl Alcohol
CNPF	-	EMBRAPA National Centre of Forestry Research
CTAB	-	Cetyltrimethylammonium Bromide
DNA	-	Deoxyribonucleic Acid
DBH	-	Diameter at Breast Height
EMBRAPA	-	Brazilian Agriculture Research Corporation
ERSC	-	EMBRAPA Research Station in Caçador
ES	-	Environmental Services
FOM	-	Ombrophilous Mixed Forest
IUCN	-	World Conservation Union
NGOs	-	Non-Governmental Organizations
GMO	-	Genetically Modified Organisms
NC	-	Not Converged
PES	-	Payment for Environmental Services
PPGEBB	-	Post-Graduation Program in Bioprocess Engineering and Biotechnology
PVPP	-	Polyvinylpyrrolidone
REDD	-	Reducing Emissions from Deforestation and Forest Degradation
SGS	-	Spatial Genetic Structure
SSR	-	Simple Sequence Repeats
UFPR	-	Federal University of Parana
UTFPR	-	Federal University of Technology – Parana

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CHAPTER I: SCOPE OF THESIS

1 INTRODUCTION

Although biotechnology is perceived as a modern science, for thousands of years humankind has used biotechnology in medicine, agriculture and food production (VERMA *et al*., 2011). Today, plant biotechnology encompasses many related areas, such as functional genomics and proteomics, molecular genetics, physiology, biochemistry and cell biology, with applications using molecular markers, hybridizations, mutations and transgenic approaches (VERMA *et al*., 2011). The widespread use of molecular markers has created a new level of genetic information from many key species in endangered ecosystems, resulting in updated strategies available for conservation of many genetic resources (KUMAR *et al*., 2011). Among the molecular markers available, the microsatellite regions (simple sequence repeats - SSR) have a strong discriminatory power and as such are a popular tool in population and conservation genetics studies (CHASE *et al*., 1996).

The aim of this study was to distinguish the patterns of genetic variation of the forest species *Araucaria angustifolia*, from varying types of Araucaria Forest remnants located near the EMBRAPA Research Station in Caçador (ERSC), Santa Catarina State. The goal was to evaluate the ecosystem services provided by different forest types in terms of the genetic repository of the species. The *A. angustifolia* tree species was selected to carry out this study as it is an endangered species and it defines the ecology of the Araucaria Forest. Moreover, it is considered a keystone species in Southern Brazil that local people recognize and identify with culturally. In the region surrounding the ERSC, native forests are restricted mostly to small fragments in different secondary stages of succession, with the exception of the ERSC where Araucaria Forest can be found in primary stages (Lacerda *et al*., 2012).

The complexity of themes addressed in this study required the structuring of this thesis in the following chapters:

Chapter I – This chapter presents a brief literature review of the main topics addressed in this study as well as a detailed description of the materials and

methods used. The literature review discusses the importance of molecular markers applied to plant biotechnology, the relationship between environmental services and ecological studies, and the relevance of the *A. angustifolia* species and the Araucaria Forest biome in Southern Brazil. The materials and methods describes the study area, the sampling strategy, as well as the molecular techniques and statistical analyses used in this study. The subsequent three Chapters (II, III and IV) present three original articles that have been submitted to journals in Forest Biotechnology.

Chapter II – This technical scientific communication describes a new, reliable, and cost effective methodology to collect samples from tall trees, aiming to isolate DNA from vascular cambium tissue, an easily accessible tissue. This article was submitted to *Revista Árvore* (Impact Factor 0.433) in December, 2013. It was revised based on the editor's recommendations in February of 2014 and it is currently in line to be reviewed by external assessors.

Chapter III – In this second scientific article, we evaluate the genetic diversity and mating system parameters of open-pollinated seeds collected in the four contrasting populations sampled in this study and carefully selected in the ERSC neighbouring region. This article was submitted in June of 2014 to *Conservation Biology* (Impact Factor 4.355).

Chapter IV – In this third scientific paper we estimate the rate of gene flow in two of the studied populations, which are located adjacent to one another, but are distinct. We also discuss data from the analysis of Spatial Genetic Structure (SGS) of adult *A. angustifolia* trees found in one of these sites where a census was carried out. This article was submitted in February of 2014 to *Tree Genetics and Genomes* (Impact Factor 2.978), revised based on the reviewers' recommendations in April 2014, and is currently under final review.

Chapter V – The final chapter highlights the main scientific findings of this thesis and their implications in evaluating environmental services provided by remnants of Araucaria Forest in Southern Brazil.

2 OBJECTIVE

The main objective of this study was to use molecular markers to understand the consequences of forest fragmentation and different types of land use on the dynamics (gene flow) and spatial distribution of genetic variation of *Araucaria angustifolia.* The aim was to evaluate the environmental services provided by forest remnants, as well as provide a suitable basis for designing conservation strategies that prioritize the sustainable use of this forest genetic resource.

3 LITERATURE REVIEW

3.1 PLANT BIOTECHNOLOGY

The wide concept of "biotech" or "biotechnology" encompasses a vast range of procedures (and history) for modifying living organisms according to human needs, going back to the domestication of animals and the cultivation of plants and microorganisms (VERMA *et al*., 2011). Among these applications, plant cultivation may be viewed as one of the earliest biotechnological enterprises. For thousands of years, early farmers used selective breeding to improve production of crops and livestock and to produce enough food to support a growing population (SRIVASTAVA *et al*., 2004).

In selective breeding, medicinal plants and forest species with desirable characteristics are selected and mated with the best suited crops, to produce offspring with the best characteristics of both parents. As large-scale agriculture became increasingly difficult to maintain, over the last several decades crop plant breeding programs have made use of artificial selection and hybridization. Modern biotechnological approaches to genetic engineering, associated with cell and tissue culture, have been used to develop the market of genetically modified organisms (GMO) and their by-products that can effectively fertilize, restore nitrogen, and control pests (SRIVASTAVA et al., 2004).

The use of molecular markers has played an increasingly important role in plant biotechnology, both for evaluating biodiversity, as well as preventing bio-piracy (KUMAR *et al*., 2009). Molecular markers have emerged as the most reliable tool for indexing genetic polymorphism of any organism, revealing polymorphism at the DNA level. The level of polymorphism detected may vary with respect to important features, such as genomic abundance, locus specificity, reproducibility, technical requirements, and financial investment (SRIVASTAVA *et al*., 2004). The choice of the most appropriate genetic markers depends on the specific application or question to be addressed, the presumed level of polymorphism, the presence of sufficient technical facilities and know-how, time constraints and financial limitations of each

study (FERREIRA; GRATTAPAGLIA, 1996). Among the many molecular markers available, the microsatellite regions have been the most widely employed to evaluate diversity due to their ease of use through simple PCR, followed by a denaturing gel electrophoresis for allele size determination, and the high degree of information provided by the large number of alleles per locus (SCRIBNER; PEARCE, 2000).

3.2 MICROSATELLITE MARKERS

There are many known molecular markers that can be applied to crop and forest species to investigate population diversity, including DNA fingerprinting and genetic mapping. Among them, the microsatellite or simple sequence repeats (SSR) is the most suitable for genetics studies (CHASE *et al*., 1996; ASHLEY, 2010). This marker is co-dominant, allowing for the possibility of discriminating between homozygote and heterozygote individuals. Baker (2000) cites a broad range of applications for this class of markers, e.g. parentage and mating systems, population genetics and conservation. In this class of markers there is a very high rate of mutation. As such, this may allow a high level of polymorphism to be detected in species whereas other markers may only detect low levels of diversity (SCOTT *et al*., 2003).

Because of the high allelic diversity, SSR regions have strong discriminatory power and are useful for the detection of recent events in population dynamics, becoming a popular tool in population and conservation genetic studies (CHASE *et al*., 1996). Among other applications, studies involving microsatellite markers are being used to describe the difference between populations and define population subdivisions. When management is an important issue, information about population subdivisions becomes an important point (SCRIBNER; PEARCE, 2000). In summary, this category of molecular marker has become an integral tool of empirical population genetics, ecology and evolutionary biology.

By the year 2000, many forest geneticists were looking for and applying microsatellite techniques to trees species. Microsatellite markers are ideal as genetic markers in determining gene flow through pollination and seed dispersal. This is because of the high exclusion probability for paternity assignment and owing to their

co-dominant inheritance and high polymorphism (DOW; ASHLEY, 1998; LIAN *et al*., 2001). Due to the high polymorphic nature of these markers, new inferences could be made, including: identification of immigrants into populations; classification of relationships (e.g. full-sibling versus parent-offspring); fine-scale gene flow; and diversity comparisons (RITLAND; RITLAND, 2000).

3.3 ENVIRONMENTAL SERVICES

The environmental services (or ecosystem services) concept relates to services provided by natural ecosystems, such as watershed quality and soil protection, maintenance of biodiversity and scenic beauty, carbon sequestration, among many others (PAGIOLA *et al*., 2007). Environmental services also encompass benefits associated with different types of actively managed ecosystems, such as sustainable agricultural practices and rural landscapes. In this context, payment for environmental service (PES) is defined by Wunder (2005) as a voluntary transaction, where a well-defined environmental service (ES) is 'bought' by an ES buyer from an ES provider, for the adoption of land uses and practices that maintain the defined services. The basic logic of PES mechanisms is shown in FIGURE 1.

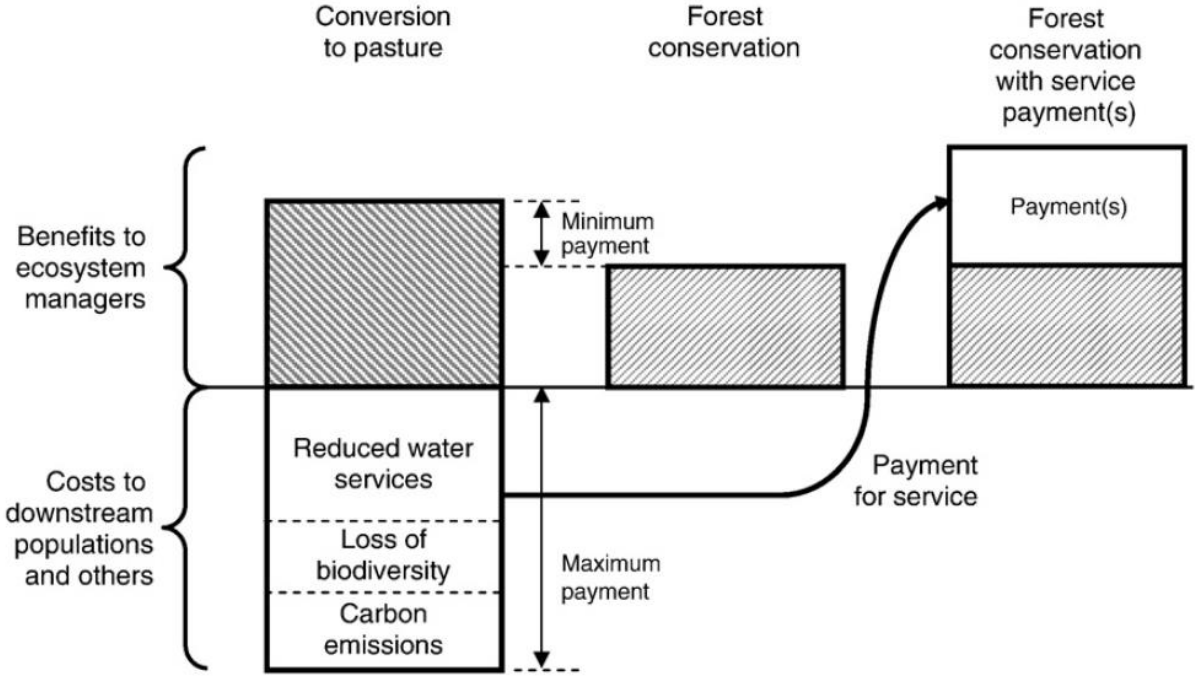

FIGURE 1 – THE LOGIC OF PAYMENTS FOR ENVIRONMENTAL SERVICES.
SOURCE: Pagiola and Platais (2007).

Chapter I

Ecosystem managers, whether they are farmers, loggers, or administrators of protected areas, often receive few benefits from land uses, such as forest conservation. The benefits of conservation are generally less than the benefits they would receive from alternative land uses, such as conversion to crop land or pasture. However, deforestation can inflict costs not only on downstream populations, who no longer receive the benefits of services such as water filtration, but also on the global community, because of reductions in biodiversity and carbon storage (ENGEL *et al*., 2008). A heightened interest in PES has emerged because international climate change discussions envisaged such schemes as an important mechanism to provide local incentives for reducing emissions from deforestation and forest degradation (REDD), resulting in the conservation of ecosystems and enhancement of carbon stocks (FERRARO, 2008).

Although REDD has followed a distinct trajectory to the implementation of PES, which is tied to the international climate change policy arena, there are important connections between REDD and PES (FERRARO, 2008). To pay for carbon fixation, developed countries provide conditional financial incentives to developing countries to undertake forest conservation measures in order to deliver localised incentives and benefits to forest users and managers (ANGELSEN *et al*., 2008). Non-governmental organizations (NGOs) frequently function as intermediaries between buyers and sellers in programs at smaller scales, although examples of this type can also be found at larger scales. Most government-financed programs rely on annual allocations through the normal budgetary process, but some have dedicated funding sources through earmarked user fees. Developed-country programs sometimes receive funding from several levels of government, while developing country programs can receive donor funding. Among user-financed programs, the classic program in both developing and developed countries involves a single buyer and a single-service (PATTANAYAK *et al*., 2010). The lines between user- and government-financed PES programs are unclear and many programs are in fact hybrids, mixing government and user financing.

According to Engel *et al*. (2008), there are at least three necessary conditions for the design of a PES scheme: a) the relationship between the type of land use being promoted and the provision of the ecosystem service must be clear; b) stakeholders must have the possibility to terminate the contractual relationship (it is a voluntary transaction); and c) a monitoring system must accompany the intervention,

in order to ensure that the provision of services is taking place (additionally and conditionality of payments).

In terms of the impact on livelihood, PES income is generally a modest contributor to participants' household financial capital, though in several cases PES revenues are considered an important supplement to household budgets, particularly for poorer households (PATTANAYAK, S. K.. *et al*., 2010). Where PES contracts are made with groups, the provision of infrastructure and services also contributes positively to physical capital. Broadly, the schemes have positive effects on human and social capital through capacity building, particularly where PES schemes work with existing community organizations (PAGIOLA *et al*., 2005). Despite the positive environmental outcomes reported by several PES schemes, the establishment of eligibility criteria and monitoring are weak points for many programs (ENGEL *et al*., 2008). The definition of eligibility criteria must be individually elaborated for each case because the provision of ES benefits is site-specific and may vary according to the biome characteristics (FERRARO, 2008). In this sense, our study aims to evaluate the patterns of genetic diversity of the key species *Araucaria angustifolia* in a wide range of landscape scenarios, to define eligibility criteria to determine which rural properties with Araucaria Forest in Southern Brazil should be chosen to receive the benefits of PES.

3.4 THE ARAUCARIA FOREST

Until the 20th Century, the Araucaria Forest biome dominated the landscape in Southern Brazil (FIGURE 2), covering an area of approximately 200,000 square kilometres (FUPEF, 2002). Primary Araucaria Forest is made up of a mixture of species, usually with three canopy layers in which the species composition may vary regionally (LEITE, 1994). The uppermost layer consists of the crowns of old *A. angustifolia* trees which allow the penetration of a considerable amount of light. The middle canopy layer is characterized by other valuable hardwood species of the Lauraceae family and the lowermost canopy by species of the Myrtaceae family and *Ilex paraguariensis*, the "erva-mate" (BITTENCOURT *et al*., 2005).

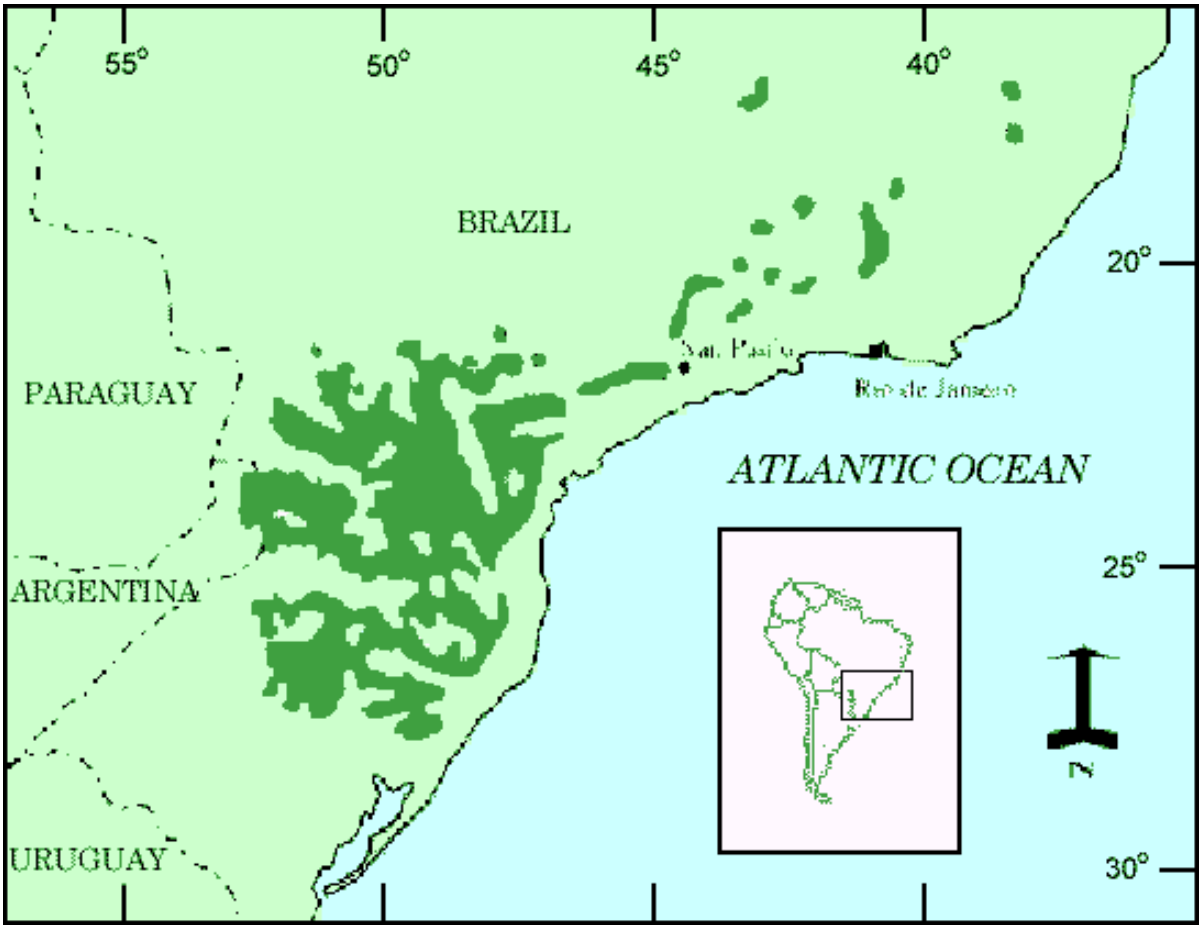

FIGURE 2 - ARAUCARIA FOREST DISTRIBUTION IN SOUTH AMERICA.
SOURCE: Kershaw and Wagstaff (2001).

According to Koch and Correa (2010), the Araucaria Forest was one of the most heavily exploited forest types in Brazil. In the second half of the 19th Century the continental lands in South America began to be gradually occupied by European colonizers (KOCH; CORREA, 2010), but the penetration of the Araucaria Forest was not significant until the beginning of the First World War. A study of the Contestado region (Southwest of Parana and North of Santa Catarina States) reported that some areas of Araucaria Forest could produce more than 500 m^3 of wood per hectare, with 428 m^3 coming only from *A. angustifolia* wood (THOME, 1995). This level of production is almost two times greater than a typical timber production in the Amazon Forest of 215 m^3 of wood per hectare (THOME, 1995).

Historically the first contact between colonizers and the Araucaria Forest was established through European colonization in the XIX Century (KOCH; CORREA, 2010). The early settlers of that region were essentially cattle farmers, who preferred grassland to forest because cattle production was the one of most important economic activities of the time (BITTENCOURT *et al*., 2005). As forest clearing

began, settlements were established and houses, furniture, and fences were produced for the farmers using *A. angustifolia* wood; the remaining forest was commonly burned to open new areas for “shifting” agriculture (MATTOS, 1972; VOLTONI, 2000).

Furthermore, during the First World War the *A. angustifolia* wood industry grew and exports were abundant, taking into account the internal collapse of wood supply in other markets (MATTOS, 1972). Around 1920, the lumber companies in Parana state supplied the markets in São Paulo, Rio de Janeiro, and Buenos Aires. During this time, the transportation system improved to become faster and more efficient with new railroad connections linking São Paulo and Rio Grande do Sul. As such, the Araucaria Forest became the major economic activity in Parana. This is known as the Parana Pine economic cycle (KOCH; CORREA, 2010).

In 1945, after the Second World War, the arrival of new migrants changed the characteristics of colonization in Southern Brazil. The Araucaria Forest became a major resource for social and economic development, providing high quality timber for general construction, furniture and long-fibre cellulose (CARVALHO, 1994). At this time, the activities of lumber companies together with newly established rural villages and urban expansion, contributed to the logging of 3.3 million *A. angustifolia* trees in a span of thirty years (from 1945 to 1975), achieving a hardwood volume of 23 million cubic meters (VOLTOLINI, 2000).

Thus, since the beginning of commercial exploitation, the practices adopted to harvest *A. angustifolia* were not inspired by a philosophy of sustainable use. The transformation of most Araucaria Forest into pastures and agricultural lands caused the extirpation of many natural populations. In 1993, the Brazilian government introduced a new legislation (*Decreto-lei* n. 750) aiming to establish rules for sustainable management of Araucaria Forest. In Parana State, only trees with more than 40 cm of DBH (diameter at breast height) could be logged, leaving at least ten mother-trees per hectare. Later, the harvesting of *A. angustifolia* was prohibited by law in 2001, when surveys showed that less than 3% of the original Araucaria Forest cover remained as fragmented forests (FUPEF, 2002; CASTELLA; BRITEZ, 2004).

Awareness is growing in Brazil that *A. angustifolia* is vulnerable. Several geographic populations were lost during colonization and as the result of development processes that occurred many years ago. Currently, the Brazilian government is promoting several initiatives to protect the Araucaria Forest. The

current legislation prohibits the harvesting of naturally regenerated *A. angustifolia* trees (PN-COMANA 278, July 18, 2001). More recently, the federal government selected five large regions in Southern Brazil to establish new conservation units using the innovative biodiversity corridors concept (PN-MMA 507, December 20, 2002). However, financial constraints have limited the Brazilian government's ability to buy rural properties in which Araucaria Forest remnants are located.

3.5 FOREST FRAGMENTATION

It is now accepted that one of the major threats to the survival of natural biomes is habitat fragmentation. The fragmentation of forest into small patches diminishes habitat area and increases patch isolation (WATTS *et al*., 2005). Forest fragmentation shapes the original forest into ecosystems that may be functionally limited compared with the original (YOUNG; CLARKE, 2000). Changes in the surrounding land use may intensify the effects of forest fragmentation by modifying connectivity among patches resulting in increased isolation (WATTS *et al*., 2005). One of the consequences of habitat fragmentation is the loss of genetic variability. However, some authors call attention to the fact that not all fragmentation leads to genetic losses (YOUNG *et al*., 1996; DUDASH; FENSTER, 2000).

The understanding of the impact of landscape fragmentation on population dynamics of key species is vital to evaluate the potential longevity and sustainability of threatened biomes (THRALL *et al*., 2000). According to Thrall *et al*., (2000), despite the fragmentation process which converts a large continuous biome into several small isolated remnants, the number of habitat patches and their size and degree of clumping can influence the functional connectivity among them. The increase of gene flow among population fragments can alleviate unfavourable genetic consequences of forest fragmentation. Corridors linking habitat fragments can re-establish gene flow between isolated patches (FRANKHAM *et al*., 2005). Additionally, restoration plans that may emerge from metapopulation theory of key species can enhance the regeneration potential of semi-natural systems (THRALL *et al*., 2000).

The conservation of genetic resources is one the most important applications of molecular ecology which, when combined with principles of landscape ecology, can be used to develop more effective conservation and restoration strategies (BAKER, 2000; FREELAND, 2005). Molecular ecology can inform projects related to landscape decision making, particularly when combined with other information sources that may help to minimize the loss of genetic variability of relevant species (FREELAND, 2005). However, the methodological difficulties of integrating landscape and genetic approaches remain a major challenge to the conservation of key species (FRANKHAM *et al*., 2005).

3.6 THE *Araucaria angustifolia* SPECIES

Araucaria angustifolia (Bert.) Kuntze, commonly known as the “Parana Pine” or *pinheiro*, is a long-lived, dioecious, conifer species, endemic to the subtropical Brazilian highlands in Southern Brazil and small patches in Argentina and Paraguay (REITZ; KLEIN, 1966). This species is the only representative of the Araucariaceae family in Brazil and its distribution is predominantly at altitudes between 500 and 1800 m (REITZ; KLEIN, 1966). Since the end of the last Century, this species was classified as vulnerable on the IUCN Red List of Threatened Species (MARTINELLI; MORAES, 2013). The Red List is extensively used by scientists as a resource for conservation-based research, as well as for formulating laws and policies on the harvesting, marketing and use of plants and biomes in need of protection.

The species has significant ecological, economic and social value and the seeds of *A. angustifolia* are an important food source for fauna, including birds and rodents, which are the main means of seed dispersal for this species. Besides being a dominant tree, populations of adult *A. angustifolia* create a microcosm in which shade tolerant plant species of other taxa can grow and develop (AULER *et al.*, 2002).

The *A. angustifolia* tree features a rectilinear, even, cylindrical trunk. Its height varies between 25-50 meters, and the trunk may range between 1-2 meters diameter at breast height (CARVALHO, 1994). The average age for an *A. angustifolia* tree with a 75 cm diameter is 150 years (VOLTOLINI, 2000). Its leaves

are hard, sharp-pointed, perennial, and remain attached to the tree for many years. When young, the tree is symmetrical, cone shaped, with branches from base to summit; mature trees are clear of branches for most of the lower part of tree and the canopy becomes chalice shaped (BITTENCOURT *et al*., 2005).

On the basis of photosynthetic and growth responses of *A. angustifolia* trees under different light conditions (EINIG *et al*., 1999), the tree is well-adapted to moderate shade. However, since there is little information on the species behaviour under natural light conditions, along with the absence of seedlings in some shaded environments, there is a commonly-held belief that *A. angustifolia* is a sun-loving, pioneer species that will not regenerate in forest under story (BITTENCOURT *et al*., 2005).

A. angustifolia is usually dioecious, rarely monoecious and presents 2n=26 chromosomes (DANNER *et al*., 2013). Like other conifers the species is wind pollinated and pollination occurs between September and October (SOUSA, 2000). Generally, the seed cones begin maturing only three years after pollination. However, the complete cycle, from primordial carpel to seed, takes four years (SOUSA, 2000; BITTENCOURT *et al*., 2005). The male catkins are dense, cylindrical, solitary or in clusters, consisting of numerous spirally-arranged stamens. Cones are broader than they are long, globular or ovoid, with woody and closely overlapping scales, which fall when the seeds are mature. Sousa and Hattemer (2003) observed that the pollen of this species is the largest of the coniferous wind pollinated trees. Additionally, the high density of forest stands in Araucaria Forest could limit pollen flow between and within populations. *A. angustifolia* seeds are bright brown and young trees generally begin to produce seeds between 12 and 15 years of age.

A. angustifolia seeds are dispersed mainly from May to August (CARVALHO, 1994). Seeds are heavy and are primarily dispersed by authochory under the canopy of the seed-tree; however, they can be subsequently dispersed by animals (birds, rodents and other mammals). Foraging animals, such as jays (*Cyanocorax caeruleus*), rodents (*Dasyprocta azarae*), domestic pigs (*Sus domesticus*), and peccaries (*Tayassu pecari*), disperse the heavy seeds (BITTENCOURT *et al*., 2005). According to Bittencourt *et al*., (2005), the seeds have a longevity of no longer than six weeks and they germinate as soon as they fall from the mother tree.

3.7 GENETIC DIVERSITY OF *Araucaria angustifolia*

Using non-adaptive markers based on growth traits, many genetic studies have been conducted aiming to characterize diversity within and among *A. angustifolia* populations. Describing morphological traits, several authors have identified genetic variation across the natural range of the species. For example, Reitz (1966) described nine botanical varieties of *A. angustifolia* based on ripening time and seed colour (MATTOS, 1972). Variation was also detected within and among three natural populations (KAGEYAMA; JACOB, 1980). *A. angustifolia,* collected from five Brazilian states, showed statistically significant differences in wood production when quantitative traits were measured (MONTEIRO; SPELTZ, 1980).

Several authors have described genotypic variation within this species among distant populations and they attribute the existence of geographic varieties in the species to its wide distribution, which contributes to this differentiation (KAGEYAMA; JACOB, 1980; STEFENON *et al*., 2007). A review of non-adaptive genetic variations within and among natural *A. angustifolia* populations can be found in “I IUFRO Meeting on Forestry Problems of the Genus Araucaria” (KAGEYAMA; JACOB, 1980).

However, the diffusion of biochemical and molecular markers that has occurred since the end of last Century has enabled many authors to evaluate new diversity levels of the species. Biochemical and molecular markers are neutral, allowing researchers to access a level of variation beyond growth traits and they can be used in a variety of applications, such as population genetics of forest species, conservation, and breeding purposes (KUMAR *et al*., 2009). Despite a total of 26 studies carried out since 1997 using different kinds of molecular markers providing detailed information about population structure within species (TABLE 1 and supplementary material), the efforts for species conservation have mostly been restricted to the enactment of legislation. However, the legislation does not take into consideration the results of the genetics research that has been conducted to date to inform conservation and management practices of *A. angustifolia*. The main objectives of these studies were to understand the status of local genetic diversity,

spatial structure, gene flow and effectiveness between physical and functional forest connections.

The general results from these studies (summarized data, TABLE 01) show that diversity levels inside specific populations are commonly relatively high (all studies); however, distant populations have specific diversity levels that are not found in other sites (18 studies). Additionally, the patterns of seed dispersal in forest remnants leads to a spatial genetic structure, where the probability of nearby trees producing progenies of half-sibs due to mating among related individuals is high (7 studies).

Moreover, seeds produced from trees developed in exploited forests can result in a high level of inbreeding due to mating among relatives. The patterns of pollen dispersion by wind allow gene flow to be higher in a forest with open canopy than inside a conserved forest with a dense canopy. Nevertheless, the diversity inside a conserved forest is higher than in an exploited fragment. Substantial gene flow has been mapped at distances of around 2 km (BITTENCOURT; SEBBENN, 2007), enabling the existence of functional connections between neighbouring Araucaria Forest stands.

This ecological and genetic information indicates that isolated female trees in the landscape located near forest remnants are important for seed collection purposes because they are able to capture the genetic diversity present in a wide region. In this context, it is important to design long-term conservation strategies based on an understanding of the effects of fragmentation on the genetics of key species (YOUNG; CLARKE, 2000; FREELAND, 2005) in threatened biomes, such as the *A. angustifolia* species in the Araucaria Forest.

TABLE 1 - QUANTITATIVE SUMMARY OF *Araucaria angustifolia* GENETICS STUDIES USING MOLECULAR MARKERS

Kind of population	Number of studies	Diversity analysis		Purposes		Advanced analysis	
		Between sites	One site	Conservation	Breeding	SGS	Gene flow
Adult trees	14	10	4	12	2	2	2
Seeds	6	6	-	4	2	-	2
Both	6	2	4	4	2	5	5
Total	26	18	8	20	6	7	9

SOURCE: The author.

4 MATERIAL AND METHODS

4.1 STUDY AREA AND SAMPLING STRATEGY

The studied landscape is dominated by small-scale farms (agriculture and pasture) and much larger areas with commercial pine plantations managed by forest companies. In this context, native forests are restricted mostly to small Araucaria Forest fragments in different secondary stages of succession, with the exception of the EMBRAPA Research Station in Caçador (ERSC), where the Ombrophilous Mixed Forest (FOM) can be found in primary stages. In the ERSC, *Araucaria angustifolia* dominates the canopy in clusters associated with others valuable hardwood trees typically from a mature FOM remnant, i.e. *Ocotea porosa*, *Cedrela fissilis*, and the economically important non-timber species *Ilex paraguariensis* (LACERDA *et al*., 2012).

The ERSC covers an area of 1,157 ha and it is located in the municipality of Caçador, mid-west Santa Catarina State (25°32'29.64" S and 50°33'44.58" W). The ERSC is mostly covered by forests (94%) that have received no silvicultural intervention after the areas was officially designated as a protected area approximately 30 years ago. Before this period, selective logging of varying intensities followed the historic context described above, although the most intense period of exploitation ceased by the mid XX Century. Most ERSC fragments are secondary forests or remnants of heavily logged forests, that have been recovering naturally for at least 30 years.

In the ERSC and its surrounding region, four contrasting sites were carefully selected to compare contemporary genetic parameters of *A. angustifolia* seeds (FIGURE 3). The first site is located inside a continuous Araucaria Forest (“Continuous”) in the ERSC. Historic records suggest that the only logging that occurred in this forest was more than 100 years ago. The second site, also inside the ERSC, is a physically isolated 7.2 ha Araucaria Cluster (“Cluster”), logged about 40 years ago and located about 2 km from Continuous population. The third site is an agricultural field abandoned 30 years ago, called the Open area (“Open”), that is adjacent to the Cluster but outside the ERSC. The fourth site is in a 2.8 ha forest

fragment inside a private rural property (“Private”), located approximately 5.5 km from the Cluster and Open sites, and 7.3 km from the Continuous population. This fourth site was selected because it is isolated by at least 0.5 km from the nearest Araucaria Forest fragment and the history of the site is very well known; the area was logged intensely approximately 50 years ago. The Open and Private sites are typical of non-forest conditions, while the Continuous and Cluster sites represent two Araucaria Forest scenarios with distinct land use histories.

To evaluate the contemporary genetic differences and similarities among these four contrasting populations, seeds from an average of ten seed trees were collected per site, and 28 seeds were analyzed per *A. angustifolia* fruit collected (280 seeds from ten seed trees in the Continuous; 364 seeds from 13 seed trees in the Cluster; 210 seeds from eight seed trees in the Open; and 259 seeds from ten seed trees in Private).

Moreover, to evaluate the rate of gene flow in the Open and Cluster areas, a census of all adult *A. angustifolia* trees was carried out in the Cluster. All 295 adult trees present in this site had their DBH, geographic coordinates and sex identified, and a vascular cambium sample was collected for DNA analysis.

FIGURE 3 – STUDY AREA IN THE ERSC NEIGHBOURHOOD.
Green: seed trees.
Red: adult trees.
SOURCE: The author.

4.2 DNA ISOLATION

The isolation of DNA from *A. angustifolia* seeds followed the protocol described Mazza and Bittencourt (2000), while the collect of vascular cambium samples and DNA isolation followed the procedures described in the Chapter II. Quantification of DNA isolated was performed comparing 5 µL of the DNA from each sample with 5 µL of Phage Lambda DNA size marker with concentrations of 20, 50 and 100 ng in agarose gel 2%. After quantification, each sample was diluted with autoclaved milli-Q water to a final concentration of 10 ng/µL and the tests with SSR markers were started.

4.3 MICROSATELLITE LOCI

The genetic estimations of all samples were made using microsatellite markers or simple sequence repeats (SSR), labelled with fluorescent dyes to detection in semi automatized Genetic Analyzer. These molecular markers are commonly used in population genetic studies, because they are codominant and exhibit high levels of variability, being suitable either for gene flow and parentage analysis, as genetic diversity studies (CHASE *et al*., 1996; SEBBENN, 2006).

Starting from 20 pairs of microsatellite markers for *Araucaria* species available, eight were selected to be amplified in three multiplex systems (Table 1 – supplementary material Chapters III and IV), using Qiagen Multiplex PCR Master Mix. Among eight SSR primer selected, two amplified unspecific regions segregating as Mendelian pattern (Aang28b and CRCAc1b), and these regions were used to increase the genetic information.

The samples were genotyped in an Automated Genetic Analyzer ABI 3500xL (Applied Biosystems), using the capillarity size standards ROX GS 500 or LIZ GS 600. The sizes of amplified fragments (alleles) were determined using the GeneMapper v.4.1 software (Applied Biosystems) and the values referring to the size of the alleles were exported to a spreadsheet to statistical analysis.

4.4 STATISTICAL ANALISYS

4.4.1 Analysis of Genetic Diversity and Fixation Index

The genetic diversity of all samples was estimated in progeny level and sites arrays. The diversity parameters estimated were the total number of alleles (k), observed heterozygosity (H_o) and expected heterozygosity at Hardy-Weinberg equilibrium (H_e). Because the sample size of adults and seeds were slightly different among sites and within progenies, we also calculated the allelic richness (*R*) using a rarefaction method (EL MOUSADIK; PETIT, 1996).

The level of inbreeding in the adults and seeds were estimated using the fixation index (*F*). For seeds, the intra-individual fixation index was calculated using reference allele frequencies estimated from the adult trees, using the SPAGeDI 1.3 program (HARDY; VEKEMANS, 2002). The significance of the *F* values was calculated using the permutations of the alleles among individuals (1,000) and a sequential Bonferroni correction for multiple comparisons (95%, α= 0.05). Except for the analysis of the intra-individual fixation index in seeds, all other analyses were run using the FSTAT program, version 2.9.3.2 (GOUDET, 2002). The t-test was used to determine if there are significant differences among the populations for *A, R,* H_o, H_e and *F*.

4.4.2 Analysis of Intrapopulational Spatial Genetic Structure

The intrapopulational spatial genetic structure (SGS) was determined for all established *A. angustifolia* trees located in the Clump, using the average coancestry coefficient (θ_{xy}) between pairs of adult trees, as described by Loiselle *et al*. (1995) in the SPAGeDI 1.3 program (HARDY; VEKEMANS, 2002). To visualise the SGS, θ_{xy} values were averaged in 15 m distance classes (0-150 m) which were then plotted

against the distances classes. To test whether there was significant deviation from a random structure, the 95% confidence interval (CI) was calculated for each observed value and each distance class based on 1,000 permutations of individuals among the distance classes.

To compare the SGS between the adult *A. angustifolia* trees, the statistic S_p (VEKEMANS; HARDY, 2004) was calculated as $S_p = -b_k / (1 - \theta_1)$, where θ_1 is the average coancestry coefficient calculated between all the pairwise individuals within the first distance class (0-15 m), and b_k is the slope of the regression of coancestry coefficient on the logarithm of spatial distance (0-150 m). To test for SGS, the spatial position for each individual was permuted (1,000 times) to obtain the frequency distribution of b_k under the null hypothesis that θ_1 and ln (d_{xy}) are not correlated.

4.4.3 Analysis of Historical Gene Dispersal from SGS

The historical gene dispersal for adults and seedlings was estimated from the SGS with the assumption that the observed SGS represents an equilibrium isolation-by-distance pattern (HARDY *et al*., 2006). The neighbourhood size (*Nb*) was estimated as $Nb = -(1-\theta_1)/b_k$ (VEKEMANS; HARDY, 2004), where b_k is the regression slope within the distance class of $\sigma_g < d_{ij} < 20\sigma_g$. This estimate of *Nb* is dependent on the assumed value for effective density, D_e (HARDY *et al*., 2006). Thus, D_e was estimated as $D_e = D(N_e / N)$, where the effective density is the ratio of the effective size to the census population size (VEKEMANS; HARDY, 2004).

Based on other plant studies (HARDY *et al*., 2006), we used *D*/10 and *D*/2 as minimum and maximum estimates of D_e. In fixing D_e, the lower and upper boundaries for the 95% confidence interval (CI) of *Nb* were estimated as $Nb_{(lower)} = (\theta_1 - 1)/(b_k - 2SE_b)$ and $Nb_{(upper)} = (\theta_1 - 1)/(b_k + 2SE_b)$, respectively, where SE_b is the standard error of b_k, calculated by jackknifing data across each loci (Hardy *et al*. 2006). The 95% CI of σ_g was estimated as $\sigma_g = \sqrt{N_b / 4\pi D_e \hat{r} D_{ep}}$ using the lower and upper *Nb* boundaries (HARDY *et al*., 2006). According Hardy *et al*. (2006), when $b_k < SE_b$, the upper boundary must be reported as infinite (∞). We also assumed the effective density as the actual density ($D_e = D$).

4.4.4 Mating System Analysis

The mating system was analyzed from seeds on the basis of mixed mating model and correlated mating model, implemented in the Multilocus MLTR program, version 3.4 (RITLAND, 2002). The calculated parameters at progeny and population levels were: multilocus outcrossing rate (t_m); single-locus outcrossing rate (t_s); outcrossing rate among related trees ($t_m - t_s$); and multilocus paternity correlation ($r_{p(m)}$). The 95% confidence interval (95% CI) of each parameter was calculated after 1,000 bootstrap replicates within progenies.

From multilocus paternity correlation ($r_{p(m)}$) the effective number of pollen donors was estimated as: $N_{ep} = 1/r_{p(m)}$ (RITLAND, 1989); meanwhile the average coancestry among plants within progenies (Θ) was calculated as: $\Theta = 0.125(1+F_p)(1+r_{p_{(m)}})$, where F_p is the coefficient of inbreeding in the parental generation, assumed to be null in a natural Araucaria forest (SOUSA *et al*., 2005). Additionally, to know the genetic structure within each progeny, the variance effective size was estimated as: $N_e = 0.5/\{\Theta[(n-1)/n]+(1+F_o)/2n\}$ (COCKERHAM, 1969), where *n* is the sample size and F_o is the coefficient of inbreeding in the progeny arrays.

The number of seed-trees (*m*) necessary to seed collect aiming to retain the reference effective population size ($N_{e(reference)}$) of 150 (LACERDA *et al*., 2008), was calculated following the method of Sebbenn (2006) as: $m = N_{e(reference)}/N_e$, based on the relation between the effective population number goal of the conservation program ($N_{e(reference)}$) and the variance effective size estimated for the average of seed trees. Finally, in order to investigate how the parameters N_{ep}, Θ and N_e affect the K, R, H_o and F within progenies, we evaluated their correlation in the SAS program (SAS, 1999), using the Spearman-ranking coefficient of determination (R^2).

4.4.5 Analysis of Variance Effective Size

The variance effective size of the reproductive population was calculated from seeds as: $\hat{N}_e = 0.5/\Theta$ (COCKERHAM, 1969), where Θ is the average group coancestry. For sexed adults, the average group coancestry was estimated as: $\hat{\Theta} = \sum_{x=1}^{n_f}\sum_{y=1}^{n_f}\hat{\theta}_f / 4n_f^2 + \sum_{x=1}^{n_m}\sum^{n_m}\hat{\theta}_m / 4n_m^2 + \sum_{x=1}^{n_f}\sum_{y=1}^{n_m}\hat{\theta}_{fm} / 2n_f n_m$ (LINDGREN; MULLIN, 1998); where θ_f, θ_m, and θ_{fm} are the coancestry coefficient between females, males, and females and males together, respectively; while n_f and n_m are the number of female (152) and male trees (143). The pairwise coancestry coefficients were estimated using the J. Nason method described by Loiselle *et al*. (1995) in the SPAGeDI 1.3 program (HARDY; VEKEMANS, 2002).

4.4.6 Paternity Analysis

The combined non-exclusion probability of parent pair and genetic identity were calculated using the CERVUS 3.0 program (MARSHALL *et al*., 1998; KALINOWSKI *et al*., 2007). The cryptic gene flow, or the probability of assigning a candidate mother or father inside the population when the true father is outside of the population, was calculated based on Dow and Ashley (1996). For this analysis, we considered all 143 male adult trees to be parent candidates when assigning the seeds of the seed trees. Parentage analysis was conducted by maximum-likelihood paternity assignment (MEAGHER, 1986) based on the multilocus genotypes of the 364 seeds collected inside the forest cluster, 210 seeds collected from the open forest, their respective maternal genotypes, and all 143 adult male trees present in the cluster where a sensus was carried out.

The most likely parent pairs were determined by the Δ statistic (MARSHALL *et al*., 1998) using the reference allele frequencies calculated in the adult population (MEAGHER; THOMPSON, 1987). Significance for Δ was determined using paternity tests simulated by the software (critical Δ), which used a confidence level of 80%,

genotyping error ratio of 0.01, and 10,000 repetitions. The calculation of critical Δ values was based on the assumption that 70% of the candidates sampled were located within the plot. If a father candidate or parent pair had a Δ value higher than the critical Δ value calculated by simulations, it was considered to be the true parent or true parent pair.

The pollen immigration rate (*m*) was calculated as the proportion of seeds that had no pollen parents ($n_{immigrant}$) inside the population relative to the total number of sampled seeds (n_{total}): $m = n_{immigrant} / n_{total}$ (BURCZYK *et al*., 1996). As all sampled individuals had a known spatial position, the distance of pollen dispersal was based on the position of the putative seed tree in relation to the putative pollen donor. To investigate whether reproductive success was a function of the distance between trees, we compared the frequency distribution of pollen dispersal with the frequency distribution of distance between all adult *A. angustifolia* trees using the Kolmogorov-Smirnov test (SOKAL; ROHLF, 1995).

The effective pollination neighbor area (A_{ep}) was calculated by taking a circular area around a seed tree, $\hat{A}_{ep} = 2\pi\hat{\sigma}^2$ (LEVIN, 1988), where σ_p^2 is the axial pollen dispersal variance. It is important to note that the parameter A_{ep} corresponds to the circular area in which 63% of pollen donors that crossed with a seed tree are expected to be located (LEVIN, 1988). The circular pollination radius was estimated as: $\hat{r}_{ep} = \sqrt{\hat{A}_{ep} / 3.1415}$ (AUSTERLITZ; SMOUSE, 2001).

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6 SUPPLEMENTARY MATERIAL

TABLE 1 - SCIENTIFIC ARTICLES THAT USED MOLECULAR MARKERS TO APPRAISE *Araucaria angustifolia* GENETIC DIVERSITY.

Authors	Molecular markers			Kind of population appraised	Sampling	Aims of study			
	Marker	Evaluated	Used			Diversity	SGS	Gene flow	Breeding
Mazza *et al*. (1997)	RAPD	120	50	5 sites in Parana State (PR)	200 trees (40 T/S)	adult trees between sites	-	-	-
Hamp *et al*. (2000)	Sequence	1	1	13 sites in 6 States (RS, SC, PR, SP, RJ, MG)	378 seeds from 15 trees (25 S/T)	progenies between sites	-	-	-
Shimizu *et al*. (2000)	Allozyme	9	7	1 site in Parana State (PR)	120 trees	adult trees inside site	-	-	-
Auler *et al*. (2002)	Allozyme	80	9	9 sites in Santa Catarina State (SC)	328 trees	adult trees between sites	-	-	-
Sousa *et al*. (2002)	allozyme	15	5	3 sites in 3 States (PR, SC, SP)	1400 seeds from 70 trees (20 S/T)	progenies between sites	-	-	-
Medri *et al*. (2003)	RAPD	500	35	3 sites in Santa Catarina State (SC)	72 trees (24 T/S)	adult trees between sites	-	-	-
Sousa and Hattemer (2003)	allozyme	7	7	3 sites in 3 States (PR, SC, SP)	1400 seeds from 70 trees (20 S/T)	progenies between sites	-	X	-
Stefenon *et al*. (2003)	AFLP	7	7	1 site in Santa Catarina State (SC)	30 trees	adult trees inside site	-	-	
Stefenon *et al*. (2004)	RAPD	40	4	2 sites in 2 States (SC, SP)	80 trees (30-50 T/S)	adult trees between sites	-	-	-

continues

TABLE 1 - SCIENTIFIC ARTICLES THAT USED MOLECULAR MARKERS TO APPRAISE *Araucaria angustifolia* GENETIC DIVERSITY.

continuation

Authors	Molecular markers			Kind of population appraised	Sampling	Aims of study			
	Marker	Evaluated	Used			Diversity	SGS	Gene flow	Breeding
Sousa *et al*. (2004)	allozyme	24	7	3 sites in 3 States (PR, SC, SP)	1400 seeds from 70 trees (20 S/T)	progenies between sites	-	-	X
Sousa *et al*. (2005)	allozyme	7	7	3 sites in 3 States (PR, SC, SP)	583 trees (199-370 T/S) + 2280 seeds from 180 trees (10-35 T/S, 8-20 S/T)	adult trees and progenies between sites	X	X	X
Salgueiro *et al*. (2005)	SSR	57	8	3 sites in Parana State (PR)	6 trees (2 T/S)	development SSR markers	-	-	-
Mantovani *et al*. (2006)	allozyme	16	7	1 site in São Paulo State (SP)	334 trees + 420 seeds (30 S/T)	progenies inside site	X	X	-
Bittencourt and Sebbenn (2007)	SSR	8	8	1 site in Parana State (PR)	397 trees + 240 seeds from 12 trees (20 S/T)	adult trees and progenies across landscape	X	X	-
Schlogl *et al*. (2007)	RFLP	17	6	8 sites in 2 States (PR, SC)	141 trees (12-20 T/S)	adult trees between sites	-	-	-
Schmidt *et al*. (2007)	SSR	50	29	5 sites in Santa Catarina State (SC)	16 trees (1-5 T/S)	development SSR markers	-	-	-
Stefenon *et al*. (2007) (2 papers)	AFLP+SSR	1 +5	6	6 sites in 4 States (PR, RS, SC, SP)	384 trees (62-64 T/S); same data to 2 papers	progenies between sites	X (1)	X (2)	X
Stefenon *et al*. (2008)	AFLP+SSR	1 +5	6	10 sites in 3 States (PR, RS, SC)	512 trees (24-64 T/S)	adult trees between sites	-	-	-

continues

TABLE 1 - SCIENTIFIC ARTICLES THAT USED MOLECULAR MARKERS TO APPRAISE *Araucaria angustifolia* GENETIC DIVERSITY.

conclusion

Authors	Molecular markers			Kind of population appraised	Sampling	Aims of study			
	Marker	Evaluated	Used			Diversity	SGS	Gene flow	Breeding
Bittencourt and Sebbenn (2008)	SSR	8	8	1 site in Parana State (PR)	108 trees + 200 seeds from 10 trees (20 S/T)	adult trees and progenies across landscape	X	X	-
Bittencourt and Sebbenn (2009)	SSR	8	8	1 site in Parana State (PR)	690 trees + 500 seeds from 25 trees (20 S/T)	adult trees and progenies across landscape	X	X	-
Patreze and Tsai (2010)	SSR + sequence	6 + 1	7	1 site in São Paulo State (SP)	105 trees	adult trees inside site	-	-	-
Ferreira *et al*. (2012)	allozyme	9	9	4 sites in Santa Catarina State (SC)	400 trees (100 T/S)+ 805 seeds from 29 trees (0-11 T/S, 28 S/T)	adult trees and progenies between sites	-	-	X
Danner *et al*. (2013) (2 papers)	SSR	8	8	11 sites in 3 States (PR, RS, SC)	306 seeds from 17 trees (18 S/T, 1-4 T/S); same data to 2 papers	progenies between sites	-	X (1)	X (2)
Santanna *et al*. (2013)	SSR	9	9	1 site in Santa Catarina State (SC)	512 trees	adult trees inside site	X	X	-

(T/S) = trees per site; (S/T) = seeds per tree; DIVERSITY RESULTS FROM ADULT TREES: inside site; between sites; landscape = different conditions inside same site; DIVERSITY RESULTS FROM SEEDS: progenies: among seeds from same mother-tree and between seeds of different mother-trees; SGS RESULTS: spatial genetic structure appraised from adult trees; BREEDING RESULTS: studies that appraised commercial populations or have breeding purposes.

SOURCE: The author.

CHAPTER II: SAMPLING FOREST TREES FOR GENETIC STUDIES - VASCULAR CAMBIUM STORAGE AND EFFICIENCY

1 ABSTRACT

Genetics studies of tree species using molecular markers usually involve a large number of samples and both the low-cost protocols for DNA extraction and commercial kits were developed for use with leaf tissue samples. However, leaf sampling of tall trees is inherently labour intensive, time demanding, costly and risky. In this context, the objectives of this study were firstly to conduct a comparative analysis of the efficiency (quality, quantity and “shelf-life”) between leaf and lignified tissue samples as sources for DNA analyses, the effects of different storing methods and their “shelf-life” on DNA quality and quantity. For the best alternative tissue, were tested variations in the extraction protocol frequently used for isolating genomic DNA from *Eucalyptus* leaves. The results showed that the sample storage method have a significant impact on the quantity of DNA obtained along time, and the reduction of salt quantity during the extraction process makes possible to obtain DNA amounts equivalent to leaves, allowing the use of vascular cambium for genetic studies. Our results also showed that process of vascular cambium sampling was at least two times faster than collecting samples from leaves. Our results demonstrate that vascular cambium sampling is an effective alternative to leaf-based sampling, and additionally to a higher efficiency, vascular cambium sampling also is safer and less expensive than leaf tissue sampling. This way, new genetic studies in forest trees can take advantage of this reliable and cost effective methodology.

Key-words: DNA isolation, wood tissue, efficiency, trees sampling.

2 INTRODUCTION

Sampling of perennial plants from natural and commercial populations for genetic studies, including genetic improvement studies, usually requires a relatively large number of samples (SHEPHERD *et al*., 2002). Both the low-cost protocols commonly used for genetic studies of perennial plants and the commercially available kits use leaf tissue as source for DNA isolation (DOYLE; DOYLE, 1990; FERREIRA; GRATTAPAGLIA, 1998; ROMANO; BRASILEIRO, 1999; MAZZA; BITTENCOURT, 2000; ZUCCHI, 2002). However, collect leaves especially in tropical and subtropical forests is a difficult task, requiring a well-trained climbing team with specialized equipment and a thorough risk assessment. As a result, collecting leaves from tall trees is a time-demanding, costly, and risky activity that has a low rate of productivity. Therefore, the need for an alternative to leaf tissue sampling for genetics studies is paramount (ARRIEL *et al*., 2002; BITTENCOURT, 2007). In this context, alternatives such as vascular cambium and bark sampling are attractive because of the simple procedures involved in sample collection and year-round access to the biological material thus rendering it a much cheaper, safer and faster alternative to leaf sampling.

Along with the logistical difficulties in collecting leaves, some species have inherent characteristics that can impose further difficulties on sample collection. For species that are deciduous or species that occur in hazardous or difficult to access environments (i.e. wetlands, steep slopes), the process of collecting leaves might be temporarily or even permanently prohibited (RAVEN *et al*., 2001). As DNA is the basis for genomics research, the procedures for its preparation should produce extracts sufficiently pure in order to minimize the effects of proteins used by molecular techniques (ROMANO; BRASILEIRO, 1999). The use of commercial kits for DNA isolation is only feasible in cases where a small number of samples is to be analysed or in cases in which DNA extraction is based on tissues collected for different purposes, such as from plant exsiccates (ASIF; CANNON, 2005) or timber tracking (DEGUILLOUX *et al*., 2002; RACHMAYANTI *et al*., 2006; TNAH *et al*., 2012). On the other hand, in studies in which a large number of samples are fundamental, the use of low-cost protocols for DNA extraction is necessary (KHAN *et al*., 2004).

Despite the crescent number of studies that recently have used vascular cambium samples as DNA source (e.g. DEGUILLOUX *et al*., 2002; COLPAERT *et al*, 2005; RACHMAYANTI *et al*., 2009; NOVAES *et al*., 2009), no study to date provides a description of the procedures and logistics that allow a simple and efficient method for obtaining samples in the field. In the main studies describing wood collection to DNA isolation purposes (COLPAERT *et al*, 2005; NOVAES *et al*., 2009), the cambium samples are preserved under refrigeration, as usually is made to leaf samples, but kept in a solution with a third part of CTAB 2% and other two parts of ethanol to avoid the freezing of cambium samples. As such, our goal was to compare different sampling methods and to evaluate best practices related to storage and "shelf-life" of samples in order to develop a simplified standard protocol for cambium sampling and the subsequent analysis of tree species genetics. Thus, the main objective of this paper is to develop a standard methodology for sampling, storing and extracting genomic DNA from vascular cambium tissue and bark as low-cost alternatives to leaf sampling. We first conducted a comparative analysis of the efficiency (DNA quality, quantity and "shelf-life") between leaf, bark and vascular cambium from *Eucalyptus* samples as sources of DNA analyses. Furthermore we optimized a DNA extraction protocol for best alternative tissue and report the comparison of their sampling efficiency with traditional leaf sampling.

3 MATERIAL AND METHODS

The wood samples were collected using a circular leather awl and a hammer. Hammering the awl into the tree trunk produces a circular wood sample. This sample can easily be separated in layers, thus enabling the collection of a sterile sample in relation to contact with potential external contaminants. Vascular cambium sampling from *Eucalyptus* trees were initially carried out following the practices established for leaf sampling, in which samples were stored in paper envelopes and kept frozen (-20ºC) until DNA extraction be performed using a low cost procedure (CTAB 2%).

The CTAB 2% methodology recommends that foliar tissue is frozen using liquid nitrogen and subsequently macerated with a mortar and pestle; however, due to the levels of lignification of the vascular cambium tissue, the suggested freezing

process hinders maceration. In order to overcome this difficulty, the samples were put in a pestle with 600 µL CTAB 2% solution allowing for the start of an early cell lysis and were then macerated; note that the CTAB 2% solution is normally used only after the leaf samples are macerated.

Initially we tested if the ontogenic phase (adults trees - DBH > 40 cm and young trees - DBH < 5 cm; DBH - Diameter at Breast Height) has an impact on the quality and quantity of DNA extracted from leaf, bark and cambium tissue of *Eucalyptus* trees using two different lengths of storage (1 day and 3 weeks). Later we tested different storing methods aiming at defining a procedure that would prevent the process of wood oxidation and consequent DNA degradation. In our subsequent tests we stored vascular cambium in plastic bags, paper envelopes containing silica gel crystals, and plastic centrifuge tubes with CTAB 2% for different periods of time (1 day, 1 week, 1 month) before DNA extraction.

After defining an acceptable longer-term storage method we tested variations of the CTAB DNA extraction protocol described by Ferreira and Grattapaglia (1998) aiming at producing large quantities of genomic DNA. The variations in the protocol involved changes in the CTAB 2% buffer components' concentrations that are used in the initial steps of the extraction process, as well as alterations in the precipitation phase, including the use of alcohol and other products for DNA cleaning. Specifically for the CTAB buffer, we tested the effects of adding the PVPP component to the buffer solution and increasing the antioxidant β-mercaptoethanol. Finally, the variations in the precipitation phase involved testing different types of alcohols (used after the last cleaning with CIA) and also the inclusion of an additional cleaning step with NaCl at the end of the protocol.

The concentration of DNA in the samples obtained from our tests was estimated using the Electrophoresis Documentation and Analysis System – EDAS 290 (KODAK, 2000), by comparing 5 µL of the DNA from each sample with 5 µL of Phage Lambda DNA size marker in concentrations of 20, 50 and 100 ng. Thirty minutes after the gel (agarose gel 1%) electrophoresis began, the gel was dyed with ethidium bromide for visualisation. The results from the software estimates were divided by 5 in order to obtain a DNA concentration in ng/µL and the total DNA amount obtained was estimated by multiplying the DNA results from EDAS software (given in ng/µL) by 50 (see Appendix 1 for the optimized protocol).

The classification of quality of the DNA obtained (high, medium and low) is

related to purity patterns obtained after quantification using the absorbance wave length of 260 nm and 280 nm (SAMBROOK; RUSSELL, 2001). The ratio among these two wave length (A260/A280) reflect the purity of sample. Samples are considered of high quality if values are higher than 1.80, medium quality for values between 1.79 and 1.50, and low quality corresponds to values of less than 1.50.

Finally, in order to compare the efficiency (in terms of time) between leaf sample collection and vascular cambium collection we recorded the amount of time a team of three people spent to collect samples, from the moment of arrival at a target tree (which includes unpacking, preparing the collection equipment, collecting and storing a sample) until the team is ready to leave (including re-packing). We recorded the time spent to collect samples for each of the three study species (*Araucaria angustifolia*, *Cedrela fissilis* and *Eucalyptus* in planted and natural forests) during one week of field work per species. The significance of all comparisons tested (p-value) was carried out using F-test for multiple factors followed by a pairwise comparison of means (Tukey-Kramer test).

4 RESULTS

Initially we tested quantity and quality of the products obtained from DNA extraction of vascular cambium and bark (adults and young trees) as an alternative to leaf tissue extraction. The results showed that DNA extraction from leaf tissue allowed for significantly higher levels of DNA than vascular cambium and bark for both storage periods, independently of the ontogenic stage ($p<0.01$): DNA extracted from leaves reached a quantity of 100 ng with very low levels of impurity (TABLE 1).

Additionally, except for bark tissue, the ontogenic stage showed no significant differences between tissue types for the storage periods considered ($p>0.05$). The extractions based on vascular cambium of young trees conducted one day after sampling were a slightly lower than 50 ng, while for adult trees the extractions were above 50 ng with no significant difference between the results ($p>0.05$); in both cases the DNA extracted contained some levels of impurity and drag (FIGURE 1).

TABLE 1 - DNA EXTRACTION QUANTITICATION OF *EUCALYPTUS* TREES FOR TWO STORAGE PERIODS CONSIDERING THE TISSUE SAMPLE TYPE AND ONTOGENIC STAGE (SAMPLES KEPT IN SILICA GEL).

Factors (A, B, C, D)	Tissue type (A)	Ontogenic stage (B)	DNA quantity (ng/µL) Storage time		Interaction factors	
			1 day (C)	3 weeks (D)	p-values	
p-values	p<0.001*	p>0.05ns	AB x p<0.001*	p<0.001*	(A)x(B)	(AB)x(CD)
	Foliar	young	120a	90a	p>0.05ns	p<0.05*
		adult	120a	90a		
	Cambium	young	45b	20b	p>0.05ns	p<0.01*
		adult	60b	20b		
	Bark	young	30c	5c	p<0.05*	p<0.001*
		adult	0d	0c		

* significant at α=0.05.
ns: not significant.
a,b,c,d – columns with identical letters represent means that did not differ statistically based on pairwise comparison (Tukey-Kramer Test; p<0.05).
SOURCE: The author.

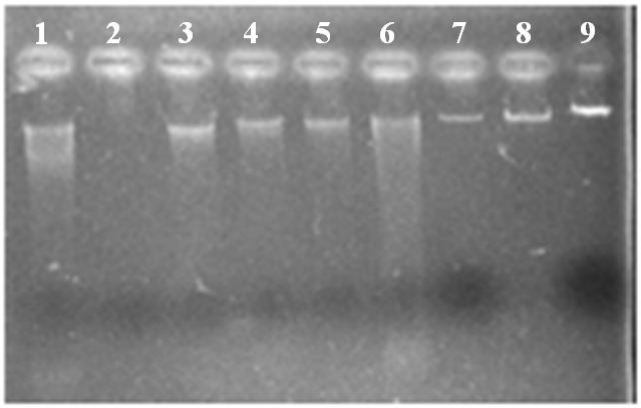

FIGURE 1 - RESULTS OF DNA ISOLATION OBTAINED FROM DIFFERENT TISSUE TYPES ANALYSED ONE DAY AFTER COLLECTION (SLOT 1, 2 AND 3: DNA FROM LEAF, BARK AND VASCULAR CAMBIUM OF AN ADULT TREE; SLOTS 4, 5 AND 6: DNA FROM LEAF, BARK AND VASCULAR CAMBIUM FROM A YOUNG TREE; SLOT 7, 8 AND 9: DNA LAMBDA PHAGE (Λ) STANDARDS OF 20 NG, 50 NG E 100 NG).
SOURCE: The author.

Although initial results showed vascular cambium as a viable alternative to foliar tissue as a source of DNA, the storage time before extraction significantly affected the quantity of DNA available. After 3 weeks of storage, vascular cambium samples kept in paper envelopes with silica gel resulted in significantly lower quantities of DNA (from 50 ng to 20 ng; $p<0.05$) and higher levels of impurity, a decrease apparently related to length of storage. Note that the bark samples only resulted in usable quantities of DNA from young trees after one day of storage (TABLE 1); after three weeks the quantity and quality of DNA obtained with low-cost protocol were inadequate for genomic studies, independent of ontogenic stage.

In relation to the influence of different storing techniques on DNA quality and quantity obtained from vascular cambium, the results showed a significant difference for DNA in terms of storage type and also a significant difference for storage time (TABLE 2). Specifically, DNA extracted one day after collection shows statistically similar results regardless of the storage method (50 ng, minimal drag). For the extractions performed one week after sampling, the results showed that only storage in plastic centrifuge tubes with CTAB 2% and paper envelopes with silica gel were able to maintain a quantity of DNA similar to that after one day of storage. The plastic bag technique showed significantly lower DNA levels when compared with plastic storage tubes but statistically similar results to paper envelopes. This is a consequence of the natural process of tissue oxidation and its subsequent degradation that is unavoidable when paper envelopes or plastic bags are used to store dry samples without freezing.

TABLE 2 - DNA EXTRACTION QUANTITICATION OF VASCULAR CAMBIUM TISSUE CONSIDERING LENGTH OF STORAGE AND STORAGE TYPE.

Factors (A, B) p-values	Storage time (A) p<0.001*	Storage type (B) p<0.001*	DNA quantity (ng/µL)	Interaction (A) x (B) p<0.001*
		Paper envelope	50[a]	
	1 day	Plastic bag	50[a]	
		Tube (CTAB2%)	50[a]	
		Paper envelope	40[ab]	
	1 week	Plastic bag	37[b]	
		Tube (CTAB2%)	50[a]	
		Paper envelope	15[c]	
	1 month	Plastic bag	15[c]	
		Tube (CTAB2%)	50[a]	

* significant at α=0.05.
[a,b,c] – columns with identical letters represent means that did not differ statistically based on pairwise comparison (Tukey-Kramer Test; p<0.05).
SOURCE: The author.

Finally, DNA extraction carried out one month after sampling showed more varied results between storage methods: plastic tubes with CTAB 2% maintained the DNA quantity levels (no significant difference across storage length) with negligible difference in drag, whereas the other two methods showed a proportional deterioration of quality related to length of time in storage and a significant reduction in DNA quantity (70% reduction in comparison to one day of storage). The results showed that storing vascular cambium in tubes with CTAB 2% is a more efficient method for DNA extraction and it allows for the DNA analysis to take place after

much longer periods in storage (e.g. more than three years based on authors tests running in the laboratory, unpublished data).

The protocol optimization using vascular cambium as a DNA source for genomic analysis (TABLE 3) began replacing the isopropanol for absolute ethanol in the first phase of precipitation with alcohol, but this change did not influence the quantity or quality of the DNA extracted. The second variation included an additional step using a cleaning solution (NaCl 1M) after the first phase of (alcohol) precipitation; such a change was designed to reduce the impurities in the isolated DNA. The results of the second protocol variation showed a sharp reduction in the DNA quantity while not improving the overall quality of the products obtained when compared to the use of the original protocol.

TABLE 3 - RESULTS OF THE CHANGES IN THE CTAB 2% DNA EXTRACTION PROTOCOL IN TERMS OF QUALITY AND QUANTITY FOR VASCULAR CAMBIUM SAMPLES (DNA QUALITY EVALUATED IN TERMS OF LEVELS OF DRAG AND IMPURITY: LOW, MEDIUM AND HIGH).

Protocol phase/component	Treatment	DNA quantity (ng/µL)	DNA quality
Precipitation phase	Isopropanol	50	medium
	Absolute ethanol	50	medium
	Cleaning with NaCl 1M	<20	high
CTAB2% buffer component	Add PVPP on buffer	50	medium
	Add 5x β-mercaptoetanol	50	medium
DNA second cleaning with CIA	no CTAB10%	>100	medium

SOURCE: The author.

As the changes in the precipitation phase were not successful, we tested variations in the components of the CTAB 2% buffer used in the initial phase of DNA extraction as a means to inhibit the effects of secondary metabolites that can degrade or immobilize the DNA, hindering the isolation. Initially we tested the addition of PVPP (polyvinylpyrrolidone) together with a fivefold increase in the β-mercaptoethanol (from 2 µL/mL to 10 µL/mL). However, those changes did not result in improvements in terms of quality or quantity of the DNA extracted. Because the only variation in the DNA quantity during the initial tests was related to the salt concentration found in the solution used for the DNA extraction, we tested the removal of the CTAB 10% buffer from the second DNA cleaning. With the removal of CTAB 10% the results showed a significantly higher DNA yield (from 50 ng to near the 100 ng level; FIGURE 2), results that are comparable to the results commonly obtained from leaf tissue in laboratory (TABLE 3).

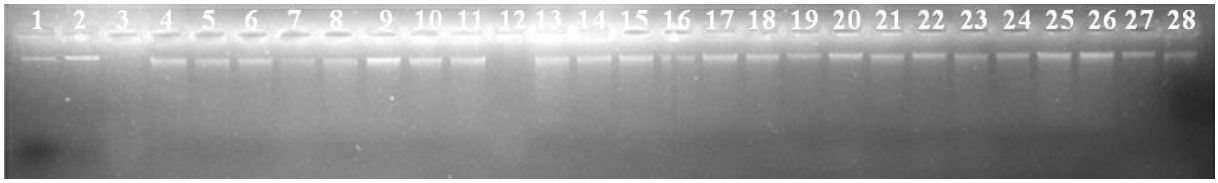

FIGURE 2 - DNA ISOLATED FROM VASCULAR CAMBIUM TISSUE SAMPLES USING THE OPTIMIZED CTAB 2% PROTOCOL (SLOT 1 AND 2: DNA LAMBDA PHAGE (Λ) STANDARDS OF 50 NG AND 100 NG; SLOT 3 AND 12: EMPTY; SLOT 4 TO 11 AND 13 TO 28: DNA OBTAINED FROM VASCULAR CAMBIUM OF ADULT TREES).
SOURCE: The author.

The use of RNAse or proteinase for RNA and protein elimination must be assessed in accordance with the quality of the DNA obtained as each species has its own particularities that might influence the process of DNA extraction. However, the application of laboratorial concepts during the sampling stage is extremely relevant to maintain the sample asepsis independent of the studied species, avoiding contamination either by another external DNA source, as selective microorganisms. Thus, the optimized protocol for DNA extraction based on vascular cambium tissue is listed below.

1- Place 0.5g of shredded vascular cambium tissue into a mortar and add 600 µL of pre-heated CTAB 2% buffer. Macerate the tissue into a homogeneuos “doughy” consistency.
2- Fill the microtube up to its conic level and water bath at 65ºC for 30 minutes; shaking every 10 minutes.
3- Let microtube cool down in room temperature and add 600 µL of CIA (24:1 chloroform isoamyl alcohol).
4- Stir samples in vortex for 5 minutes and than centrifuge microtubes for 5 minutes at 13000 rpm.
5- Transfer supernatant (about 600 µL) to new microtubes and add 600 µL of CIA for another 5 minutes of vortex stiring.
6- Centrifuge samples for 5 minutes at 13000 rpm. Transfer supernatant (about 400 µL) to new microtubes.
7- Add 500 µL of refrigerated isopropanol (4ºC) and keep microtube in freezer (-20^{o}C) for at least 2 hours (ideally overnight).
8- Centrifuge samples kept in freezer for 3 minutes at 7000 rpm.
9- Discard isopropanol carefully so pellet is not lost and add 1 mL of ethanol 70%. Keep samples in freezer for at least 1 hour.

10- Discard ethanol 70% (again being careful with pellet) and add 500 µL of ethanol absolute.
11- Homogenize by inversion (without breaking pellet) and centrifuge for 3 minutes at 5000 rpm. Discard ethanol absolute.
12- Place samples to dry at room temperature for abour 1 hour and than dissolve DNA pellets in 100 µL of TE with RNAse (2 µL of RNAse for each 1 mL of TE).

5 DISCUSSION

The low-cost protocols for DNA extraction based on CTAB recommend that the leaf tissue is frozen in liquid nitrogen for later maceration. However, because of lignification such a recommendation was not feasible for vascular cambium and bark tissues without the use of the appropriate equipment to macerate/grind those tissues. In order to overcome this difficulty, vascular cambium tissues were macerated (skipping the freezing process) with the addition of 600 µL CTAB solution allowing for the initiation of early cell lysis.

Genomic studies can be based on either foliar or vascular cambium tissues although relative differences in DNA quantity and quality are to be expected. The quality of DNA – expressed in terms of level of impurity and the existence of drag – showed that foliar tissue yields DNA with negligible levels of impurity and drag after one day of storage while cambium and bark produced higher (but still acceptable) levels of impurity for the same period. After three weeks all types of tissue produced higher levels of impurity and drag, but the foliar and cambium tissue samples were still considered satisfactory for genomic research.

The development of a DNA extraction protocol based on bark samples showed results that varied depending on the tree's ontogenic stage. Specifically, DNA extraction from the bark of adult trees did not yield detectable quantities of DNA whereas extraction from the bark of young trees yielded usable quantities of DNA (TABLE 1). One possible explanation for such a difference is related to inherent characteristics of cells at various life stages. In adult trees, samples contain a reduced amount (or an absence) of living cells that are fully differentiated (not

meristematic). In the case of young trees we suspect that there is a considerable presence of living cells and undifferentiated cells (meristematic) which are a viable source for DNA extraction.

In relation to the techniques for storing tissue samples, vascular cambium samples kept in CTAB 2% solution was the most efficient storage method among the techniques tested as it reduces tissue oxidation and subsequent DNA degradation. Although we tested DNA storage for up to three weeks, we expect that vascular cambium samples stored in CTAB should be viable for much longer periods (possibly years) without degradation or visible signs of oxidation; such a method also allows for longer storage periods under refrigeration, eliminating the need for freezing. Other advantages of using vascular cambium includes removing the need to refrigerate samples in the field as well as reducing contact with external contaminants which is a concern with foliar tissue as leaves are in constant contact to potential contaminants.

Although the results show that vascular cambium is a viable source of tissue sampling for DNA extraction, the quantity of DNA obtained might be lower than desired in some situations. By eliminating CTAB 10% from the second cleaning stage (with CIA) we managed to improve the amount of DNA as the component has a higher concentration of salt which in turn reduces the quantity of DNA at the end of the extraction process. The CTAB 10% solution is an important component in the process of obtaining DNA from leaves when there are high levels of secondary metabolites in the tissue (ROGSTAD, 1992).

The CTAB 10% solution is composed mainly of salt, CTAB and water, and this component is not listed in the original protocol described by Doyle and Doyle (1991), being introduced later during the adaptation of the protocol for genomic DNA isolation based on *Eucalyptus* leaf samples by Ferreira and Grattapaglia (1998). Although eliminating this step resulted in an increase in the DNA extracted with a slight reduction in quality, the resulting DNA extract proved to be appropriate for molecular studies both to planted or natural forests.

In relation to logistics, leaf sampling requires a team with two specialized climbers involving specific training and equipment and a third supporting team member whose activities might include clearing trails, collecting samples, identifying trees and measuring additional variables (coordinates, DBH and height of trees, etc). For vascular cambium sampling, we also used a team of three people but with a more homogenous distribution of responsabilities: one person was responsible for

tree identification and opening access to trees; the second member was responsible for cambium data collection and decortication; the third person was responsible for handling the samples (collecting, tagging and transporting). The difference between the two methods is not restricted to efficiency but also involves very different levels of risk, cost and physical effort. While leaf sampling requires two members to be exposed to significant risk while climbing, teams involved in sampling vascular cambium can have a dedicated person to collect samples (which tends to reduce errors) while others conduct much less physically demanding and risky activities. This way, vascular cambium sampling is more efficient, safer and cheaper to collect from a wide range of forest species.

6 CONCLUSION

The DNA extraction protocol and sampling method system established in this paper was tested on samples obtained from the vascular cambium of natural and planted forests: 1,500 samples of the native conifer *Araucaria angustifolia* (BITTENCOURT, 2007; MEDINA-MACEDO, unpublished data); 200 samples of native broadleaf *Cedrela fissilis* (MEDINA-MACEDO, unpublished data), and finally 1,500 samples of exotic *Eucalyptus* spp (CARVALHO, 2009; MEDINA-MACEDO, 2009; SENA, 2009). The results of our tests showed that vascular cambium is a viable alternative to leaf tissue especially when the research is focused on tree species whose leaves are mostly found in the canopy of tropical and sub-tropical forests; bark, on the other hand, is not a viable alternative. Moreover, collecting vascular cambium dramatically reduces the time required for collecting samples and it is significantly less risky when compared to climbing trees to collect leaf samples. Along with the difficulties related to the logistics of collecting leaves, some species have inherent characteristics that prevent or prohibit the process of leaf collection, including their occurrence in high-risk environments or on steep-slopes.

Although our evaluation of the efficiency of sampling methods was designed to include a variety of forest conditions and species in order to ensure its applicability in broader conditions, we must emphasize that differences are to be expected depending on specific conditions (i.e. type of forest and species studied). It is

important to note that studies on genetic diversity usually evaluate relatively small sections of the genome, which require isolated quantities of DNA samples that can be stored in small, 1.5 mL Eppendorf tubes. On the other hand, genome mapping uses a much larger section of the genome that requires larger quantities of DNA; in this case we recommend that larger amounts of vascular cambium should be collected and stored in larger plastic tubes, such as the 15mL Falcon tubes or 5 mL Eppendorf tubes.

The most commonly used method for DNA extraction from leaf tissue is a difficult task especially in tropical and subtropical forests that requires well-trained climbing teams with specialized equipment in a time demanding, costly and risky field operation. In order to find an alternative to foliar sampling, we compared different sampling methods and developed a method for DNA extraction based on vascular cambium that we expect will allow researchers to use a much faster and safer method of sampling while guaranteeing the quality and quantity of DNA required for molecular studies. Some caution should be advised in using our results for planning field-work logistics and applying the protocol developed for DNA extraction as testing with other species may be an important first step in implementing this protocol. Because of the variety of tree species considered in this study, we expect that the protocol has the potential to reduce significantly the costs and risks involved in collecting and analysing samples for molecular studies of trees in a large varying of forest conditions.

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CHAPTER III: USING GENETIC DIVERSITY AND MATING SYSTEM PARAMETERS ESTIMATED FROM GENETIC MARKER TO DETERMINE STRATEGIES FOR SEED COLLECTION TO CONSERVATION OF *Araucaria angustifolia* (BERT.) O. KUNTZE (ARAUCARIACEAE) POPULATIONS

1 ABSTRACT

In order to understand the impacts of forest fragmentation on *A. angustifolia* populations, we evaluated using SSR markers the genetic diversity and mating system of open-pollinated seeds from four populations of different sizes and spatial isolation levels around one of most conserved sites with Araucaria forest. Across the four scenarios, was identified significant positive association between N_{ep} and the total number of alleles (k) and allelic richness (R), showing that the increase in the N_{ep} also increase the allelic diversity within progeny. We found higher amount of private (25) and different alleles (113) in the pristine population (Continuous forest) than in other populations. The multilocus paternity correlation ($r_{p(m)}$) was significantly higher and effective number of pollen donor (N_{ep}) was significant lower in the most isolated site (Private), decreasing the variance effective size (N_e) of their seeds. This way, the number of seed trees required for seed collection (m) in a non-forest and isolated site (at last 0.5 km from other Araucaria forest fragment and more than 5 km from other sites) is about 25% higher than in sites closer to forest scenarios (maximum 46). In conclusion, we believe that strategies for *A. angustifolia* conservation on the landscape level should focus not only larger populations such as found in protected areas, but also owe include smaller fragments in private properties located closer (about 2 km) to larger populations, because these populations are able to maintain high levels of genetic diversity and low levels of relatedness.

Key-words: *Araucaria angustifolia*, mating system, landscape diversity.

2 INTRODUCTION

The canopy of the Sub-tropical Ombrophilous Forest of Southern Brazil, commonly called Araucaria Forest, is dominated by *Araucaria angustifolia* (Bert.) O. Kuntze (Araucariaceae). This species in turn defines the Araucaria Forest physiognomy, which is affected by an altitude-influenced climate characteristic of the region in which the species occurs (CARVALHO, 2003). The forest contains a unique mixture of temperate and tropical floristic elements (KLEIN, 1960), that is considered as a biodiversity hotspot (MITTERMEIER *et al*., 2004). Among all species that occur in that biome, *A. angustifolia* (Brazilian pine) has unique economic and cultural importance in Southern Brazil, where it predominates. The large dimensions and unique crown shape mark the region's landscape, and it has become a defining symbol of regional cultural identity (LACERDA *et al*., 2012).

Intense land conversion starting in the mid XIX Century has caused a widespread fragmentation that has significantly altered the landscape over the last two centuries. In southern Brazil, the landscape was modified from a continuous forest covering about 200,000 km^2, into a matrix dominated by agriculture in which the vast majority of forest fragments are smaller than 50 ha. Currently, large natural Araucaria Forest populations are restricted to less than 3% of its original area (LACERDA *et al*., 2012; VIBRANS *et al*., 2013).

The fragmentation of forest ecosystems is a phenomenon that affects all habited regions of the planet as a result of human activities (YOUNG; BOYLE, 2000). When fragmented populations become genetically isolated, there is an imminent risk of a loss of genetic diversity that is critical to adaptation and long-term survival of forest species (SORK; SMOUSE, 2006). Many authors evaluating genetic diversity of different forest species attributed an immediate loss of alleles, associated with a reduction in the effective population size, to forest fragmentation (NASON; HAMRICK, 1997; WHITE *et al*., 1999; JUMP; PENUELAS, 2006; SEBBENN *et al*., 2011). Evaluating Araucaria seeds in different scenarios, Bittencourt and Sebbenn (2007, 2008, 2009), Ferreira *et al*. (2012) and Danner *et al*. (2013) independently reported an increase in the relatedness and inbreeding levels related to the isolation level of evaluated seed trees, due alterations in the mating system and gene flow patterns.

The mating system and gene flow of such species determines the dynamics of genes recombination from reproductive trees within a population to a descendant population, affecting the genetic diversity within progenies, rates of outcrossing, correlated mating and mating among related individuals (SEBBENN, 2006; MORAES *et al*., 2012). The most important aspect that helps in defining the mating system is its sexual system. Dioecious species such as *A. angustifolia* reproduce sexually only by outcrossing in random or among correlated mating, where progenies present only mixtures of half and full-sibs individuals. Monoecious tree species usually have mixed mating system and their open-pollinated progenies may present mixtures of self-sibs, half-sibs, full-sibs and self-half-sibs (SEBBENN, 2006).

Moreover, Sousa and Hattemer (2003) note that because of the characteristics of *A. angustifolia* pollen (e.g. large and non-saccate), there is a tendency towarding mating between a female tree and the nearest male trees due to restricted flotation and dispersion. As such, the species highly susceptible to fragmentation. However, Bittencourt and Sebbenn (2007) reported pollen flow up to 2 km between isolated trees and the nearest forest fragment. As described for other forest species (HAMRICK, 2004; JUMP; PENUELAS, 2006), longevity combined with an effective pollen dispersal can enhance *A. angustifolia* resistance to the negative effects caused by forest fragmentation (BITTENCOURT; SEBBENN, 2007, 2008, 2009).

As the genetic diversity and mating system process within and between remnants with Araucaria Forest are still not fully understood, it is essential to examine the impacts of fragmentation on the mating system of this species. This information is critical in the design and implementation of efficient conservation strategies for the Araucaria Forest biome (BITTENCOURT; SEBBENN, 2007, 2009; FERREIRA *et al*., 2012). In order to understand how fragmentation that occurred in a region many years ago may affect the contemporary mating system patterns of open-pollinated *A. angustifolia* populations, we evaluated seeds from four distinct sites across a landscape. These sites differ in terms of density, fragment size, isolation distance and historic levels of land use and they are located in a region that contains few areas with primary Araucaria Forest in a pristine state (1%). We aim to answer the following research questions: I) Are differences in mating system parameters among the four different scenarios the result of anthropogenic intervention in the populations? II) What is the influence of population isolation, forest structure and

logging history on the genetic diversity of contemporary open pollinated progeny arrays?

3 MATERIAL AND METHODS

3.1 STUDY AREA AND SAMPLING

In the study region the landscape is dominated by small-scale farms (agriculture and pasture) and much larger areas with commercial pine plantations managed by forest companies. Native forests are restricted mostly to small Araucaria Forest fragments in different secondary stages of succession, with exception from the EMBRAPA Research Station in Caçador (ERSC) where the Araucaria forest can be found in primary stages. The ERSC covers an area of 1,157 ha located in the municipality of Caçador, mid-west of Santa Catarina State (25°32'29.64" S and 50°33'44.58" W). The ERSC is mostly covered by forests (94%) that have received no silvicultural intervention since it was designed a protected area 30 years ago. Selective logging occurred at varied intensities whithin the ERSC, although most intense exploitation ceased by the mid XX Century. Most fragments located around the ERSC are secondary forests or previously heavily logged forest remnants that have been recovering naturally for at least 30 years.

The study sample included four forest types located within and around the ERSC. We sampled and genotyped 280 seeds from ten seed trees (28 seeds per tree) from a primary continuous Araucaria Forest located in the ERSC (“Continuous”), where logging occurred more than 100 years ago. Also in the ERSC, 364 seeds from 13 seed trees (28 seed per tree) were sampled in a 7.2 ha Araucaria Cluster (“Cluster”) that was logged about 40 years ago and is located 2 km from the Continuous population. These two sites are considered as forest conditions. Adjacent to the Cluster, the Open population (“Open”) is an old agricultural field that was clear-cut at some point in the past and abandoned for at last 30 years; the distance between the naturally regenerated seed trees range from 20 to about 500 m. From this site, we sampled 210 seeds from eight seed trees (mean of 26 seeds per tree).

Additionally, we sampled 259 seeds from ten seed trees (mean of 26 seeds per tree) from a forest fragment located inside a private rural property (“Private”), located approximately 5.5 km from Cluster and Open populations and 7.3 km from the Continuous population. This fragment has an area of 2.8 ha and it is isolated from the nearest Araucaria Forest fragment by 0.5 km. The Private population represents subsequent generations after very intense logging occurred about 50 years ago. The Open and Private populations are characterized as non-forest conditions.

3.2 MICROSATELLITE ANALYSIS

The isolation of DNA from *A. angustifolia* seeds followed the protocol described Mazza and Bittencourt (2000). Quantification was performed comparing 5 µL of the DNA from each sample with 5 µL of Phage Lambda DNA size marker with concentrations of 20, 50 and 100 ng in 2% agarose gel. After quantification, each sample was diluted with autoclaved milli-Q water to a final concentration of 10 ng/µL to begin SSR markers tests. Of the 20 pairs of microsatellite markers available for *Araucaria* species, eight were selected to be amplified in three multiplex systems (TABLE 1 - supplementary material), using Qiagen Multiplex PCR Master Mix. Of the eight SSR primer pairs selected, two amplified unspecific regions segregating with a Mendelian pattern (Aang28b and CRCAc1b), and these regions were used to increase the genetic information. We genotyped the samples in an Automated Genetic Analyser ABI 3500xL (Applied Biosystems), using the capillarity size standards ROX GS 500 or LIZ GS 600. The sizes of amplified fragments (alleles) were determined using the GeneMapper v.4.1 software (Applied Biosystems) and the values referring to the size of the alleles were exported to a spreadsheet for statistical analyses.

3.3 ANALYSIS OF GENETIC DIVERSITY AND FIXATION INDEX

The genetic diversity of was estimated in level of progeny and progeny arrays. The estimated parameter were the total number of alleles (k), observed heterozygosity (H_o) and expected heterozygosity at Hardy-Weinberg equilibrium (H_e). Because the sample size was different, among the sites and within progeny, we also calculated the allelic richness (*R*) using a rarefaction method (EL MOUSADIK; PETIT, 1996). The level of inbreeding was estimated using the fixation index (*F*) and the statistical significance of the *F* values was calculated using the permutations of the alleles among individuals, associated a sequential Bonferroni correction for multiple comparisons (95%, α= 0.05). These genetic analyses and the number of private alleles in the samples were estimated using the FSTAT program, version 2.9.3.2 (GOUDET, 2002). The t-test was used to determine if there are significant differences among the populations for *A, R,* H_o H_e and *F*.

3.4 MATING SYSTEM ANALYSIS

The mating system was analysed on the basis of mixed mating model and correlated mating model, implemented in the Multilocus MLTR program, version 3.4 (RITLAND, 2002). The calculated parameters at progeny and population levels were: multilocus outcrossing rate (t_m); single-locus outcrossing rate (t_s); outcrossing rate among related trees ($t_m - t_s$); and multilocus paternity correlation ($r_{p(m)}$). The 95% confidence interval (95% CI) of each parameter was calculated after 1,000 bootstrap replicates within progenies. From multilocus paternity correlation ($r_{p(m)}$) the effective number of pollen donors was estimated as: $N_{ep} = 1/r_{p(m)}$ (RITLAND, 1989); meanwhile the average coancestry among plants within progenies (Θ) was calculated as: $\Theta = 0.125(1+F_p)(1+r_{p_{(m)}})$, where F_p is the coefficient of inbreeding in the parental generation, assumed to be null in a natural Araucaria forest (Sousa *et al*., 2005). Additionally, to know the genetic structure within each progeny, the variance effective size was estimated as: $N_e = 0.5/\{\Theta[(n-1)/n]+(1+F_o)/2n\}$ (COCKERHAM, 1969), where *n* is the sample size and F_o is the coefficient of inbreeding in the progeny arrays. The number of seed-trees (*m*) necessary to seed

collect aiming to retain the reference effective population size ($N_{e(reference)}$) of 150 (Lacerda *et al*., 2008), was calculated following the method of Sebbenn (2006) as: $m = N_{e(reference)} / N_e$, based on the relation between the effective population number goal of the conservation program ($N_{e(reference)}$) and the variance effective size estimated for the average of seed trees. Finally, in order to investigate how the parameters N_{ep}, Θ and N_e affect the K, R, H_o and F within progenies, we used the coefficient of determination (R^2) using Spearman-ranking correlation in the SAS program (SAS, 1999).

4 RESULTS

4.1 GENETIC DIVERSITY

We identified 121 alleles across the entire sample. The greatest number of alleles (k) in the Continues population (113) in relation to all other sites (TABLE 1), and this total was significantly higher than the result found for the Open Area (78). The allelic richness (R) in the Continuous population (10.5) also was significantly higher than in the Open Area (7.5). Considering the number of private alleles of each site in relation to all others sites together (TABLE 2), we found the greatest amount in Continuous (25) and the lowest in the Open (1) and Clump (1) populations.

In the Private population we identified six alleles not found in the other sites. In pairwise comparisons of private alleles of sites individually (TABLE 2), the Continuous has between 30 and 36 private alleles in relation to each of other sites, while the Cluster has between 1 and 8, Open between 1 and 4, and Private between 6 and 14 in relation to the other sites. The observed (H_o) and expected heterozygosity (H_e) not were significantly different among populations. The fixation index (F) was significant lower than zero in all populations, being for the Open Area (-0.25) significantly lower than in the Continuous (-0.14) and Cluster (-0.15) populations.

TABLE 1 - SITE HISTORY, AREA, SAMPLE SIZE, GENETIC DIVERSITY AND MATING SYSTEM PARAMETERS IN PROGENY ARRAYS OF FOUR *Araucaria angustifolia* POPULATIONS (SD IS THE STANDARD DEVIATION; ± CI IS THE 95% CONFIDENCE INTERVAL; * *P*< 0.05).

Populations	Continuous	Clump	Open	Private
Study areas characteristics				
Historical logging	Logged continuous primary forest	Logged isolated primary forest	Pasture with multi-aged natural *A. angustifolia*	Open (managed) secondary forest
Area (ha)	1,157	7.0	2.4	2.8
Number of seed trees [number of seeds]	10 [280]	13 [264]	8 [210]	10 [278]
Genetic diversity parameters	mean (±SD)	mean (±SD)	mean (±SD)	mean (±SD)
Total number of alleles: k	113	84	78	88
Allelic richness: R	10.5 ± 3.9	8.2 ± 3.2	7.5 ± 2.7	8.3 ± 2.7
Observed heterozygosity: H_o	0.83 ± 0.13	0.85 ± 0.12	0.85 ± 0.11	0.88 ± 0.11
Expected heterozygosity: H_e	0.73 ± 0.10	0.74 ± 0.09	0.68 ± 0.09	0.75 ± 0.06
Fixation index: F	-0.14 ± 0.12*	-0.15 ± 0.10*	-0.25 ± 0.11*	-0.17 ± 0.14*
Mating system parameters	mean (± CI)	mean (± CI)	mean (± CI)	mean (± CI)
Multilocus outcrossing rate: t_m	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (0.99-1.00)	1.00 (1.00-1.00)
Single-locus outcrossing rate: t_s	0.99 (0.99-1.00)	0.99 (0.99-1.00)	0.99 (0.99-1.00)	0.97 (0.96-0.99)
Mating among relatives: $t_m - t_s$	0.01 (0.00-0.01)	0.01 (0.00-0.01)	0.01 (-0.01-0.01)	0.03 (0.01-0.04)
Multilocus paternity correlation: $r_{p(m)}$	0.08 (0.03-0.09)	0.07 (0.04-0.08)	0.13 (0.03-0.16)	0.35 (0.18-0.38)
Effective number of pollen donors: N_{ep}	12.3 (11.6-33.3)	14.1 (11.9-26.3)	7.4 (6.2-30.3)	2.9 (2.6-5.6)
Coancestry within progeny: Θ	0.135 (0.129-0.136)	0.134 (0.130-0.136)	0.142 (0.129-0.145)	0.169 (0.147-0.173)
Variance effective size: N_e	3.26 (3.25-3.39)	3.40 (3.37-3.50)	3.23 (3.17-3.51)	2.70 (2.64-3.03)
Number of seed trees: m	46 (44-46)	44 (43-45)	46 (43-47)	56 (49-57)

SOURCE: The author.

TABLE 2 - PRIVATE ALLELES IN PROGENY ARRAYS FROM FOUR *Araucaria angustifolia* POPULATIONS.

Groups	Distance (km)	k_1	$k_{1(private)}$	k_2	$k_{2(private)}$	k_{shared}	k_{Total}
Continuous x All others	-	113	25	96	8	88	121
Clump x All others	-	84	1	120	37	83	121
Open x All others	-	78	1	120	43	77	121
Private x All others	-	88	6	115	33	82	121
Continuous x Clump	2	113	30	84	1	83	114
Continuous x Open	2.5	113	36	78	1	77	114
Continuous x Private	7.3	113	31	88	6	82	119
Clump x Open	0.5	84	8	78	2	76	86
Clump x Private	5.5	84	6	88	10	78	94
Open x Private	5	78	4	88	14	74	92

k_1 and k_2 are respectively the number of alleles in the 1st and 2nd terms of a comparison; $k_{1(private)}$ is the number of private alleles in the 1st term of a comparison in relation to 2nd term; $k_{2(private)}$ is the number of private alleles in the 2nd term of a comparison in relation to 1st term; k_{shared} is the number of shared alleles by all sites of a comparison; k_{Total} is the total number of alleles by all sites of a comparison.
SOURCE: The author.

At the progeny level (TABLE 3), the total number of alleles (k) ranged from 45 to 74 and the average allelic richness (R) ranged from 3.8 to 6. The observed heterozygosity (H_o) ranged among progeny from 0.78 to 0.94 and the fixation index (F) was significant lower than zero in all progeny (ranging from -0.52 to -017). These results suggest selection for heterozygous individuals in all 41 offspring evaluated in the four contrasting scenarios (TABLE 3).

TABLE 3 - SAMPLE SIZE, GENETIC DIVERSITY AND MATING SYSTEM PARAMETERS IN LEVEL OF PROGENY OF FOUR *Araucaria angustifolia* POPULATIONS (±SD IS THE STANDARD DEVIATION).

Population-Progeny	n	k	R	H_o	F_o	t_m ± SD	$t_m - t_s$ ± SD	$r_{p(m)}$ ± SD	N_{ep}	Θ	N_e
Continuous, Logged continuous primary forest, 1,157 ha											
1	28	64	5.2	0.89	-0.32*	1.00±0.00	0.03±0.01	0.14±0.05	7.2	0.142	3.23
2	28	64	5.5	0.80	-0.21*	1.00±0.00	0.03±0.01	0.05±0.01	21.3	0.131	3.47
3	28	64	5.3	0.85	-0.27*	1.00±0.00	0.04±0.01	0.03±0.01	32.3	0.129	3.52
4	28	55	4.7	0.80	-0.24*	1.00±0.00	0.05±0.01	0.07±0.02	13.3	0.134	3.39
5	28	62	5.1	0.79	-0.26*	1.00±0.00	0.04±0.01	0.05±0.02	18.9	0.132	3.45
6	28	64	5.3	0.84	-0.28*	1.00±0.00	0.05±0.01	0.04±0.02	22.7	0.131	3.48
7	28	65	5.2	0.82	-0.26*	1.00±0.00	0.03±0.01	0.09±0.04	10.9	0.137	3.34
8	28	70	6.0	0.84	-0.17*	1.00±0.00	0.01±0.01	0.03±0.01	31.3	0.129	3.51
9	28	67	5.5	0.82	-0.25*	1.00±0.00	0.03±0.01	0.03±0.01	32.3	0.129	3.52
10	28	74	5.9	0.88	-0.26*	1.00±0.00	0.08±0.01	0.07±0.02	13.9	0.134	3.40
Clump, Logged isolated primary forest, 7 ha											
1	28	67	5.7	0.88	-0.25*	0.99±0.00	0.07±0.01	0.09±0.02	11.5	0.136	3.36
2	28	61	5.3	0.92	-0.30*	0.99±0.00	0.03±0.01	0.09±0.03	11.2	0.136	3.35
3	28	55	4.8	0.85	-0.32*	0.99±0.00	0.06±0.01	0.11±0.03	9.4	0.138	3.31
4	28	62	5.3	0.83	-0.21*	0.99±0.00	0.06±0.01	0.03±0.01	30.3	0.129	3.51
5	28	62	5.3	0.86	-0.27*	0.99±0.00	0.06±0.01	0.04±0.01	25.0	0.130	3.49
6	28	64	5.4	0.87	-0.27*	0.99±0.00	0.05±0.01	0.04±0.01	27.8	0.130	3.50
7	28	61	5.4	0.87	-0.25*	0.99±0.00	0.03±0.00	0.04±0.01	26.3	0.130	3.50
8	28	59	4.9	0.80	-0.29*	0.99±0.00	0.04±0.00	0.09±0.02	11.4	0.136	3.36
9	28	64	5.3	0.84	-0.23*	0.99±0.00	0.05±0.00	0.05±0.01	19.6	0.131	3.46
10	28	64	5.4	0.87	-0.22*	0.99±0.00	0.05±0.01	0.05±0.02	19.6	0.131	3.46
11	28	50	4.3	0.82	-0.32*	0.99±0.00	0.07±0.01	0.13±0.03	7.6	0.142	3.24
12	28	63	5.3	0.83	-0.23*	0.99±0.00	0.05±0.01	0.05±0.02	20.8	0.131	3.47
13	28	60	5.4	0.85	-0.22*	0.99±0.00	0.03±0.00	0.05±0.01	18.5	0.132	3.45

continues

TABLE 3 - SAMPLE SIZE, GENETIC DIVERSITY AND MATING SYSTEM PARAMETERS IN LEVEL OF PROGENY OF FOUR *Araucaria angustifolia* POPULATIONS (±SD IS THE STANDARD DEVIATION).

conclusion

Population-Progeny	n	k	R	H_o	F_o	t_m ± SD	$t_m - t_s$ ± SD	$r_{p(m)}$ ± SD	N_{ep}	Θ	N_e
Open, Pasture with multi-aged natural *A. angustifolia* population, 2.4 ha											
1	28	66	5.5	0.85	-0.24*	0.99±0.00	0.03±0.00	0.03±0.01	28.6	0.129	3.51
2	28	45	3.9	0.84	-0.36*	0.96±0.03	0.06±0.03	0.12±0.03	8.3	0.140	3.27
3	28	55	4.7	0.81	-0.27*	0.92±0.00	0.06±0.01	0.06±0.02	16.7	0.133	3.43
4	28	38	3.8	0.86	-0.39*	0.97±0.01	0.04±0.00	0.20±0.04	4.9	0.151	2.85
5	14	48	4.2	0.86	-0.39*	0.99±0.00	0.08±0.01	0.17±0.06	5.7	0.147	3.14
6	28	53	4.5	0.88	-0.37*	0.99±0.00	0.08±0.01	0.09±0.03	10.5	0.137	3.34
7	28	49	4.3	0.84	-0.30*	0.99±0.00	0.06±0.01	0.18±0.05	5.6	0.147	3.13
8	28	44	3.9	0.86	-0.40*	0.99±0.00	0.05±0.00	0.28±0.05	3.5	0.161	2.90
Private, Open (managed), secondary forest, 2.8 ha											
1	28	56	4.9	0.93	-0.36*	1.00±0.00	0.05±0.01	0.23±0.07	4.3	0.154	3.00
2	28	57	4.9	0.87	-0.33*	1.00±0.00	0.11±0.02	0.22±0.06	4.5	0.153	3.02
3	28	54	4.7	0.92	-0.38*	1.00±0.00	0.05±0.01	0.09±0.04	11.4	0.136	3.36
4	28	47	4.1	0.91	-0.43*	1.00±0.00	0.12±0.04	0.38±0.08	2.6	0.173	2.71
5	28	40	4.0	0.94	-0.52*	0.99±0.00	0.04±0.00	0.30±0.07	3.3	0.163	2.68
6	28	54	4.6	0.87	-0.30*	1.00±0.00	0.03±0.00	0.23±0.06	4.4	0.154	3.01
7	28	54	4.7	0.88	-0.35*	1.00±0.00	0.02±0.00	0.10±0.04	9.5	0.138	3.31
8	28	46	4.3	0.78	-0.31*	1.00±0.00	0.07±0.01	0.30±0.08	3.3	0.163	2.79
9	21	47	4.1	0.83	-0.33*	1.00±0.00	0.06±0.01	0.53±0.10	1.9	0.191	2.48
10	28	58	5.2	0.83	-0.20*	1.00±0.00	0.07±0.01	0.13±0.03	7.6	0.141	3.24

n is the sample size; k is the total number of alleles; R is the allelic richness for 14 individuals; H_o is the observed heterozygosity; F is the fixation index; multilocus outcrossing rate (t_m); rate of mating among relatives ($t_m - t_s$); multilocus paternity correlation ($r_{p(m)}$); effective number of pollen donors (N_{ep}); coancestry within progeny: (Θ); variance effective size (N_e); * P<0.05.

SOURCE: The author.

4.2 MATING SYSTEM

The population multilocus outcrossing rate (t_m) was not significantly different from unity (1.0) in the four populations. The difference $t_m - t_s$ was significantly higher than zero only in the Private population (0.03), suggesting that despite the low value, the rates of mating among relatives in this site are higher than in others (TABLE 1). The multilocus paternity correlation ($r_{p(m)}$) was significantly higher than zero in all sites (ranging from 0.07 to 0.35), and significant higher in the Private population in relation to the others. Moreover, Private population showed a significantly low effective number of pollen donors (N_{ep} = 2.9) in comparison to the other three sites, where N_{ep} ranged from 7.4 to 14.1. Consequently, in the Private population the average coancestry (Θ) within progeny arrays was significantly higher than the other three sites, and the variance effective size (N_e) was significantly lower. As such, in the Private population the number of seed trees required for seed collection (m) is higher (56) than in other sites (maximum 46 seed trees).

For the individual progeny level analysis, the multilocus outcrossing rate (t_m) was significantly different from unity (1.0) for only three seed trees of the Open area (TABLE 3). Although the difference $t_m - t_s$ was significantly higher than zero for almost all progeny (with the exception of one progeny from Continuous), the estimated values were very low (maximum of 12%).This result indicates that seed trees are mating with related individuals at different intensities in all sites.

The multilocus paternity correlation ($r_{p(m)}$) was significantly higher than zero in all progeny (ranging from 0.03 to 0.53), but it was particularly high in the Private population in relation to the others sites. Consequently, in the Private population the effective number of pollen donor (N_{ep}) was generally lower, the mean coancestry (Θ) within progeny arrays was higher and the variance effective size (N_e) was lower than in other sites.

4.3 CORRELATION AMONG PARAMETERS

Significant association was detected between effective number of pollen donors (N_{ep}), coancestry within progenies (Θ) and variance effective size (N_e), and the parameters total number of alleles (k) and allelic richness (R) (TABLE 4). The parameters N_{ep} and N_e were significantly positive in relation to k and R (R^2 above 0.50), indicating that an increase in the number of pollen donors increases the total number of alleles and allelic richness within progeny. On other hand, the parameter Θ was significantly negatively associated with k and R (R^2 around -0.50), indicating that an increase in the relatedness within progeny decreases the genetic diversity.

TABLE 4 - COEFFICIENT OF DETERMINATION (R^2, DF= 39) AMONG THE EFFECTIVE NUMBER OF POLLEN DONORS (N_{ep}), AVERAGE COANCESTRY WITHIN PROGENIES (Θ), VARIANCE EFFECTIVE SIZE (N_e), TOTAL NUMBER OF ALLELES (k), ALLELIC RICHNESS (R), OBSERVED HETEROZYGOSITY (H_o) AND FIXATION INDEX (F) IN *Araucaria angustifolia* OPEN-POLLINATED PROGENIES.

	k	R	H_o	F
N_{ep}	0.502 **	0.538 **	-0.079	0.003
Θ	-0.501 **	-0.504 **	0.053	0.008
N_e	0.607 **	0.580 **	-0.074	0.003

** *P*< 0.01.
SOURCE: The author.

5 DISCUSSION

5.1 GENETIC DIVERSITY

The four studied populations showed high levels of heterozygosity regardless their differences in terms of *A. angustifolia* density and isolation, forest structure and logging history. The expected heterozygosity (H_e) levels found in this study ranging from 0.68 in the Cluster to 0.75 in the Privatepopulation were higher than those detected in other *A. angustifolia* open-pollinated progenies evaluated with SSR markers. Bittencourt and Sebbenn (2008) evaluating adult trees and seeds from a large continuous fragment (108 adults and 190 seeds considered together) described average H_e levels of approximately 0.58. Also using SSR markers, Danner *et al*. (2013) reported average H_e levels of 0.678 for open pollinated progenies, and 0.526 for hand pollinated progenies.

In relation to observed heterozygosity (H_o), only Danner *et al*. (2013), evaluating open-pollinated progenies in isolated seed trees inside an urban area, reported a high average value of 0.833. This result is the same H_o average value detected in the Continuous population in this study (the lowest average among the four populations). Of the studies that evaluated *A. angustifolia* genetic diversity using SSR markers, Danner et al. (2013) used seven of ten regions evaluated in this study (obtaining very similar results), while Bittencourt and Sebbenn (2007, 2008, 2009) used five of ten SSR regions evaluated in this study (obtaining comparable results).

Moreover, our results show that the population size as well as the level of isolation (distance to larger fragments) can influence the number of private alleles among populations. The highest values for total and private alleles were identified in the Continuous population (113 and 25, respectively), which is the largest and most well conserved population. The second highest values were identified in the Private population (88 and 6, respectively) and the lowest values were found in the Open population, a physically isolated and poorly conserved area (TABLE 2). Likewise, also found unique private alleles when comparing distant sites in a landscape that have contrasting historical use and conservation conditions. In contrast to the

traditional focus on large and continuous reserves, our results confirm Bittencourt and Sebbenn's (2009) findings emphasizing the need to integrate small fragments and even isolated trees in conservation strategies for *A. angustifolia*.

5.2 MATING SYSTEM

In general, all 41 *A. angustifolia* progenies evaluated in the four populations as well as the population estimates displayed multilocus outcrossing rates (t_m) not significantly different to unity (1.0), a typical pattern that confirm the dioecism of the species (TABLES 1 and 3). Outcrossing rates close to unity have been reported for *A. angustifolia* using distinct molecular markers in scenarios of commercial plantations, continuous and fragmented forest populations, and isolated trees (TABLE 2 – supplementary material), but the authors attributed these t_m values lower than unity to mating among relatives (minimum 0.82 using allozime markers).

Rarely monoecious trees in this species have been identified (REITZ; KLEIN, 1966; DANNER *et al*., 2013). Danner *et al*. (2013) studying the mating system of three open-pollinated progenies of monoecious trees, found a high but significantly lower than unity outcrossing rate (minimum 0.94), suggesting selfing. However, all seed trees evaluated in this study are dioecious, and therefore, the three cases where t_m was significantly lower than unity (Open population) is likely due to mating among relatives, as attributed by Sousa *et al*. (2005) in their study.

We found low levels of mating among relatives ($t_m - t_s$) at both population and progeny arrays levels (maximum 12%). Many authors attribute mating among relatives to the existence of intrapopulation spatial genetic structure (SGS), a common phenomenon in *A. angustifolia* populations resulting mainly from gravitational seed dispersal close to the seed tree (SOUSA *et al*., 2005; MANTOVANI *et al*., 2006; BITTENCOURT; SEBBENN, 2007, 2008, 2009; Ferreira *et al*., 2012; SANT'ANNA *et al*., 2013).

Mating among relatives produce inbreeding and may result in inbreeding depression. However, of the 41 offspring evaluated in this study we found no evidence of inbreeding. In fact, in all sites we found significant lower than zero fixation index (F), suggesting the existence of genetic mechanisms working to select

against inbred individuals. Furthermore, several genetic studies which evaluated adult and the subsequent generation of non-reproductive juveniles (TABLE 2 - supplementary material), attributed the null increase in the inbreeding levels across successive generations to the elimination of inbred individuals between seed and adult stages (BITTENCOURT; SEBBENN, 2007, 2008; STEFENON *et al*., 2007; PATREZE; TSAI, 2010; SANT'ANNA *et al*., 2013).

The estimated paternity correlation ($r_{p(m)}$) at the population and progeny levels showed that the *A. angustifolia* fruits are composed by mixtures of half-sibs and full-sibs seeds. Wind pollination is probably the cause of the high frequency of half-sibs within progeny, mixing pollen from different, and generally unrelated, male trees. Although the results indicate a predominance of half-sibs at the population level, the absolute offspring $r_{p(m)}$ values were lower in the forest scenarios (all under 15%) than in the non-forest sites (maximum of 53%). This result demonstrates how the level of isolation and the extent of forest conservation can affect the diversity among offspring of this species. Similar results were found in other mating system studies of the species (TABLE 2 - supplementary material). Thus, model that estimate genetic parameters for species conversation and genetic improvement must consider this mixture of relatedness within open-pollinated progeny to avoid overestimation of such parameters in successive generations (SOUSA *et al*., 2005).

Our results suggested higher frequency of random mating in forest conditions (Continues and Clump) and not isolated trees (Open). In the evaluation of the seed trees in these scenarios, we found that there is significantly greater number of pollen donors (population level: N_{ep} ranging from 7.4 to 14.1, progeny level: N_{ep} ranging from 3.5 to 32.3) than in the more isolated Private population (population level: N_{ep} = 2.9, progeny level: N_{ep} ranging from 1.9 to 11.4).

The Continuous, Cluster and Open area are close to each other and are not isolated by distance in the landscape; thus, there are many potential pollen donors. In contrast, the Private is isolated from the nearest fragments more than 0.5 km, consequently with fewer potential pollen donors than the other populations. This result indicates that the spatial isolation of populations of *A. angustifolia*, and possibly other wind pollinated tree species, may have lower effective numbers of pollen donors than in forest or non-isolated conditions. Similarly, Bittencourt and Sebbenn

(2007) found an average of 12.6 pollen donors in a small forest fragment and an average of 7.7 pollen donors for isolated seed trees in the landscape.

In a similar pattern observed for N_{ep} but with contrasting values, in the Private population the coancestry within progenies (Θ) was significantly higher, while the variance effective size (N_e) was significantly lower than in the other sites. A low number of pollen donors increases the frequency of identical by decent alleles within progeny, consequently increasing relatedness and decreasing the effective size. As such, and because the Private population has a higher Θ and lower N_e than the other populations, in the context of an isolated population with low potential pollen donors, the number of seed trees required for seed collection (m) to retain the reference effective size (150 unrelated individuals) will be higher (56) than in non-isolated populations (maximum of 46 seed trees). As the species is dioecious and the rate of mating among relatives was low, the N_{ep} is the main determining factor of the coancestry (Θ) and variance effective size (N_e) within progeny, as well as the number of seed trees necessary for seed collection (m).

Finally, as the effective number of pollen donors determines the coancestry and variance effective size within progeny, all this parameters have strong impact on the genetic diversity within progeny. Our results show that in contrasting scenarios the N_{ep}, Θ and N_e are correlated with the total number of alleles (k) and allelic richness (R) (TABLE 4). Therefore, an increase in the number of pollen donors increases the total number of alleles and allelic richness within progeny. Thus, seeds collected from seed trees in the Private population will present low allelic diversity due to pollination from a small number of pollen donors, resulting from small population size and spatial isolation of the population. The significant correlation between mating system parameters shows a clear pattern among the four contrasting scenarios, discussed further below.

5.3 DIVERSITY ACROSS THE LANDSCAPE

Although all sites evaluated in this study showed high levels of genetic diversity when compared with other studies, we observed a tendency toward higher allelic diversity in fruits from trees in larger populations with less devastating historic logging events and lower levels of spatial isolation. The isolation level of the Cluster (a large and physically isolated population, located 2 km from the Continuous forest) did not show significant genetic differences from the large Continuous forest. This indicates that the isolated forest population is either large enough (> 250 adults) and/or close enough (about 2 km) to the Continuous population to maintain similar genetic levels. Bittencourt and Sebbenn (2007), evaluating gene flow in trees surrounded by agricultural fields, identified functional connectivity among isolated seed trees and the nearest forest remnant located about 2 km away.

Populations characterized as non-forest conditions (outside the ERSC) showed distinct results as they differ in terms of their spatial isolation. Specifically, the Open population showed relatively similar genetic levels and correlated mating to the adjacent Cluster population and there is a clear influence of the larger Cluster on the Open population. On the other hand, the Private population is a small fragment physically isolated by more than 0.5 km to the nearest Araucaria Forest remnant (which is small and highly degraded) and more than 5 km from the other study populations. The Private population showed lower levels of allelic diversity in comparison to the Cluster and the Open area together (a much larger population); however, in this population we identified unique alleles not present in the other populations. The isolation level seems to have a significant impact on correlated mating and levels of genetic diversity, unless a relatively large population is located nearby. Nevertheless, populations located further than 2 km from large clusters can retain genetic diversity that differs from populations located within a 2 km radius.

Due to the existence of SGS, fruits collected from an *A. angustifolia* tree located in a forest will certainly have some levels of coancestry (SOUZA *et al*., 2005; MANTOVANI *et al*., 2006; BITTENCOURT; SEBBENN, 2007, 2008, 2009; FERREIRA *et al*., 2012). However, our results show that these coancestry levels will be lower than in isolated seed trees located in non-forest conditions. Forest scenarios seem to exert a limiting effect on progeny to deter increases of coancestry in subsequent generations. This selection is due not only to the high density of pollen donors in forests, but also to the existence of genetic mechanisms restricting mating among related individuals, as discussed above. In non-forest conditions, pollen flow

can come from longer distances; however, lower numbers of pollen donors can undermine the mechanisms that would restrict mating among relatives, despite the long distances between related individuals. In such scenarios, coancestry levels are influenced by the physical isolation of seed trees in addition to the level of relatedness with the neighboring reproductive trees. On the other hand, isolated seed trees or small populations located in proximity to larger continuous forests (or large *A. angustifolia* clusters), will tend to bear fruits with higher diversity levels and the added advantage of much easier and safer conditions for fruit collection.

In light of our results, the level of spatial isolation of a forest fragment has more of an impact on the number of pollen donors mating with the seed tree and genetic levels of a population than the size of the fragment. Even small populations can maintain high levels of genetic variation if a large population is located nearby. Therefore, in order to retain an effective size of 150 unrelated *A. angustifolia* trees for seed collection strategies, it is necessary to collect fruits from approximately 45 seed trees located in or near forest scenarios, or from 56 seed trees located in areas located further away from large populations (i.e. smaller and spatially isolated fragments).

6 CONCLUSION

Araucaria angustifolia has been the center of much debate since legal restrictions were implemented prohibiting the harvesting of the species and the species was deemed under threat of extinction. Nevertheless, *A. angustifolia* seeds are intensely commercialized as a food commodity during the fall in Southern Brazil. Efforts to conserve the species have been restricted to the enactment of legislation, which generally does not take into account the scientific information presented in genetics studies such as ours. New forms of legislation, based on genetics and conservation research, needs to be developed that promotes and enables the use, conservation, and management of *A. angustifolia* as a genetic resource. In this context, our study assessed the relationship between historical logging events, population size, and levels of isolation, demonstrating genetic diversity and mating

systems on a landscape scale. This type of information should form the basis for further conservation and management plans for *A. angustifolia*.

The relatively high allelic richness and exclusive alleles in smaller, managed (for non-timber forest products), and relatively isolated populations, such as the Private population, highlights the relevance of small and privately owned fragments for the species' conservation. Furthermore, our results also show that small populations are able to maintain high levels of genetic diversity and low levels of relatedness if located close to larger populations (i.e. Cluster). As such, we believe that strategies for *A. angustifolia* conservation should take a landscape approach and focus not only on larger populations, such as those found in protected areas, but also include smaller fragments located on private properties. The vast majority of remaining forest fragments in Southern Brazil are found on small farms. The banning of forest management (of any type) is has been an economic burden for small-scale farmers and has contributed to a limited increase in the areas with *A. angustifolia* over the past 20 years. Considering the economic and cultural relationship that exists between small-scale farmers and *A. angustifolia*, together with the importance of small fragments for the species conservation, it is crucial to develop inclusive strategies that give incentives for land owners to maintain and sustainably use their forests.

We believe that for the conservation of *A. angustifolia* and the biome's overall biodiversity, both large and small forest fragments should be maintained. Both scenarios can help in connecting the various populations and isolated trees across the landscape and both types have high and unique levels of genetic diversity. As such, if one considers genetic diversity as the main criterion in selecting areas for conservation, our results show that the combination of larger populations with smaller fragments that are not located adjacent to each other would optimize the conservation of genetic diversity of *A. angustifolia* on a landscape scale. Finally, considering the general absence of continuous large populations of Araucaria Forest remaining in Southern Brazil, our results show that focusing on smaller fragments scattered throughout the landscape but close enough to allow for pollen flow is a valid conservation strategy.

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9 SUPPLEMENTARY MATERIAL

TABLE 1 - MULTIPLEX COMBINATION OF SSR PRIMERS FROM ARAUCARIA SPECIES USED IN THIS STUDY.

SSR primer	Dye	Multiplex	Tm ºC	Repeat motif	Fragment range (pb)	Author	*Araucaria* specie	GenBank accession number
Ag 45	Fam	I	57.8ºC	$(GT)_4$	145 – 180	Salgueiro *et al*. 2005	*A. angustifolia*	AJ749966
AS 190	Ned	I	57.8ºC	$(AT)_8(GT)_{12}$	165 – 190	Robertson *et al*. 2004	*A. subulata*	AY426083
CRCAC1a	Fam	I	57.8ºC	$(GA)_{19}$	180 – 210	Scott *et al*. 2003	*A. cunninghamii*	AF522871
CRCAC1b	Fam	I	57.8ºC	$(GA)_{19}$	75 – 115	Scott *et al*. 2003	*A. cunninghamii*	AF522871
Ag 20	Fam	II	59.3 ºC	$(GA)_{12}$	225 – 260	Salgueiro *et al*. 2005	*A. angustifolia*	AJ749964
Ag 56	Ned	II	59.3 ºC	$(TC)_{11}$	135 – 160	Salgueiro *et al*. 2005	*A. angustifolia*	AJ749967
Aang 01	Hex	III	56.5 ºC	$(CT)_{22}$	200 – 235	Schmidt *et al*. 2007	*A. angustifolia*	AY865575
Aang 14	Fam	III	56.5 ºC	$(GA)_{27}$	200 – 225	Schmidt *et al*. 2007	*A. angustifolia*	AY865583
Aang 28a	Hex	III	56.5 ºC	$(CT)_{11}$	140 – 160	Schmidt *et al*. 2007	*A. angustifolia*	AY865592
Aang 28b	Hex	III	56.5 ºC	$(CT)_{11}$	160 – 175	Schmidt *et al*. 2007	*A. angustifolia*	AY865592

SOURCE: The author.

TABLE 2 - RESULTS OF MATING SYSTEM PARAMETERS REPORTED IN OTHER STUDIES OF *Araucaria angustifolia* POPULATIONS.

Parameters	Sousa *et al*. (2005) mean (min-max)	Mantovani *et al*. (2006) mean (min-max)	Bittencourt and Sebbenn (2008) mean (min-max)	Ferreira *et al*. (2012) mean (min-max)
Molecular marker used (number of loci)	Allozime (7)	Allozime (7)	SSR (8)	Allozime (8)
Multilocus outcrossing rate: t_m	0.99 (0.97-1.01)	0.96 (0.82-1.12)	0.99 (0.96–1.02)	0.98 (0.87-1.03)
Effective outcrossing rate among relatives: 1-t_s	0.05 (0.01-0.09)	0.10 (0.0–0.32)	0.12 (0.08–0.15)	0.04 (-0.07-0.09)
Multilocus paternity correlation: $r_{p(m)}$	0.29 (0.11-0.60)	0.19 (0.10-0.28)	0.20 (0.16–0.23)	0.24 (0.03-0.66)
Variance effective size: N_e	3.09 (2.50-3.62)	3.36 (3.12-3.64)	2.87 (2.79–2.96)	3.2 (2.4-3.9)

SOURCE: The author.

CHAPTER IV: HIGH LEVELS OF GENETIC DIVERSITY THROUGH POLLEN FLOW IN THE CONIFEROUS *Araucaria angustifolia*: A LANDSCAPE LEVEL STUDY IN SOUTHERN BRAZIL

1 ABSTRACT

In this study, genetic diversity, inbreeding, spatial genetic structure (SGS) and pollen dispersal are analyzed using ten microsatellite loci from two populations of the dioecious, wind-pollinated, coniferous tree *Araucaria angustifolia* in Southern Brazil. The study populations include an undisturbed seven ha *A. angustifolia* cluster, where all adult trees were mapped and sampled, and an adjacent, long-abandoned, open agricultural area with an aggregated *A. angustifolia* population. Seeds were collected from 13 seed trees inside the forest cluster and from eight seed trees in the open forest. Our results showed that the adults present high levels of heterozygosity (H_O= 0.91) and an absence of inbreeding. However, significant SGS was detected up to 90 m in the forest cluster suggesting that near-neighbor trees are related. The estimate of effective population size was lower than the total number of trees in the cluster (N_e/N= 0.19), which can be explained by the presence of SGS in the stand. Substantial external pollen flow was detected in the forest cluster (26%) and open forest (20%), indicating that the reproductive population size is greater than the sampled populations, explaining the high genetic diversity in this population. Our results indicate that this site has potential for *in* and *ex situ* conservation due to high levels of genetic diversity and gene immigration resulting from pollen flow. Conservation strategies for *A*. *angustifolia* should focus not only on forest fragments, but should also include the preservation of isolated trees throughout the landscape.

Key-words: Araucaria, genetic diversity, pollen flow, SGS, microsatellite markers.

2 INTRODUCTION

With its unique crown shape and widespread dispersion across Southern Brazil, the conifer *Araucaria angustifolia* is typical of the region's landscape. The tree is deeply embedded in the culture and culinary practices that define the region's identity. *Araucaria angustifolia* was a valuable timber species and one of the country's main exports during the twentieth century, causing a dramatic reduction in its natural population. After the species was deemed threatened with extinction and placed on the Brazilian Red List (MARTINELLI; MORAES, 2013), logging was forbidden altogether which enabled a gradual rebound of its natural populations. Although the increase in the population tends to be seen as positive for the conservation of the species, few studies have examined the levels of genetic variability in new populations and how pollen flow and other processes occur between established and new populations. To address this gap, we evaluate the genetic variability and pollen flow between a well conserved *A. angustifolia* cluster and a younger population located in proximity to the established forest. The results are expected to shed light on the importance of forest fragments, isolated trees and young forests in the conservation of *A*. *angustifolia* in Southern Brazil.

Araucaria angustifolia (Bert.) O. Kuntze (Araucariaceae) or Brazilian pine is a wind pollinated conifer. Its seeds are dispersed mainly by barochory but with some zoochory by agoutis, birds, and squirrels (CARVALHO, 2003). The species is mainly dioecious (although rare occurrences of monoecious individuals have been reported by Danner *et al*. 2013) and it typically occurs at altitudes between 500 to 2,300 m a.s.l. Because of its dominant position in the canopy, it is characteristic of the forest type which is commonly known as Araucaria Forest (Ombrophylous Mixed Forest). According to Carvalho (2003) and Mattos *et al*. (2010), *A. angustifolia* trees can live for more than 300 years, reach up to 50 m in height and 250 cm in diameter at breast height (DBH); it grows in deep, well-drained soils in areas with even precipitation distributed throughout the year. The natural distribution of *A. angustifolia* is mostly in Brazil, from the states of Minas Gerais to Rio Grande do Sul (19°15' - 31°30'S and 41°30' - 54°30'W), but it also occurs in limited areas in Argentina and Paraguay (BITTENCOURT *et al*., 2004). Before European colonization of South America, Araucaria ecosystems covered an estimated area of 200,000 km^2 consisting of

mostly continuous forests. With unmitigated exploitation and land conversion, the area covered by Araucaria forests has been reduced to an estimated 1-3% of old-growth forests (CARVALHO, 2003). In response to the dramatic reduction of its original population, different legislation and conservation efforts have been put in place that have enabled the recovery of *A. angustifolia* populations and related ecosystems. Consequently, significant *A. angustifolia* natural populations can now be found in forest fragments of varying sizes and under different secondary stages of succession; additionally it is quite common to find isolated trees in farmlands, individuals planted along property fence lines and even in landscaped urban areas.

The species presents pioneer-like behaviour in abandoned areas and grasslands but it can also act as a partially shade tolerant tree in forest environments. Because of its long life cycle, natural regeneration might be uncommon for long periods until gaps are formed in the forest canopy allowing for the development of saplings. On the other hand, the regeneration of the species has been reported to be compromised by factors such as dominance of invasive species (e.g. native bamboos) in the understory, seed and seedling predation, and seed collection for human consumption (LACERDA *et al*., 2012; KELLERMAN *et al*., 2013). The reduction of continuous habitats into small forest fragments and problems with regeneration can cause an immediate decrease in genetic diversity due to the loss of alleles (WHITE *et al*., 1999) which is associated with a reduction in population size (CASCANTE *et al*., 2002), disruptions to natural mating systems, and interruption of gene flow. In turn, these processes are linked to an increase in inbreeding levels and population divergence (JUMP; PENUELAS, 2006; BITTENCOURT; SEBBENN, 2007; SEBBENN *et al*., 2011).

The loss of genetic diversity due to forest fragmentation is a critical concern for the long-term survival of forest populations, affecting fitness and adaptability of trees inside fragmented areas (BITTENCOURT; SEBBENN, 2009). However, the impacts of forest fragmentation on a tree species' genetic diversity may not be evident immediately after fragmentation (BITTENCOURT; SEBBENN, 2009). The longevity of trees species, combined with dispersal of seed and pollen over long distances can enhance their resistance to the negative effects of fragmentation (HAMRICK, 2004; JUMP; PENUELAS, 2006). Additionally, analyses of genetic variability based on remaining established trees that carry similar levels of genetic diversity to an original population may not represent the contemporary effects of

forest fragmentation on genetic diversity (ALDRICH *et al*., 1998; KETTLE *et al*., 2007; BITTENCOURT; SEBBENN, 2009). As a response to the fragmentation of Araucaria Forest, rates of mating among relatives are expected to increase, the consequences of which can be observed in seeds through a reduction in heterozygosity and an increase in inbreeding levels (BITTENCOURT; SEBBENN, 2007, 2008, 2009). Inbreeding can lead to reduced fitness and can limit the ability of a population to adapt to environmental changes, especially in small and isolated populations (FRANKHAM *et al*., 2005; ISAGI *et al*., 2007).

The impacts of forest fragmentation on genetic diversity and inbreeding of tree species can be seen in both the short- and long-term (HAMRICK, 2004). The short-term effects are more evident in seedlings and seeds produced after fragmentation (HAMRICK, 2004), while the long-term effects are related to an increase in the level of inbreeding and genetic drift, both associated with genetic isolation due to a reduction in the gene flow caused by fragmentation (YOUNG *et al*., 1996). To assess these processes, approaches based on microsatellite markers are often used as they provide a high exclusion probability for paternity assignment due to codominant inheritance and elevated levels of polymorphism, allowing for reliable estimates of effective gene flow (DOW; ASHLEY, 1998; BURCZYK *et al*., 2004; BITTENCOURT; SEBBENN, 2007, 2008, 2009; ASHLEY, 2010).

In populations of tree species, limited seed and pollen dispersal tend to create spatial genetic structure (SGS), or a non-random distribution of genotypes within populations, where genetic relatedness will be higher among near-neighbours than with more distant conspecifics (VEKEMANS; HARDY, 2004; CARVERS *et al*., 2005). Although SGS is mainly a consequence of seed and pollen dispersal patterns (LOISELLE *et al*., 1995), there are many other factors that can interact and influence SGS in forest fragments, such as adult density, mating system, colonization history and natural selection (VEKEMANS; HARDY, 2004; JONES *et al*., 2006). For forest species with seeds dispersed by gravity (barochory), such as *A. angustifolia*, SGS within populations is expected to be higher than in species with extensive or random seed dispersal (JONES *et al*., 2006; NG *et al*., 2006). Additionally, if a substantial proportion of pollen is dispersed over short distances, mating will inevitably occur among relatives (BITTENCOURT; SEBBENN, 2009), which can reduce offspring fitness due to the occurrence of biparental inbreeding depression (STACY, 2001;

JONES *et al*., 2006). Thus, information on pollen dispersal distance and SGS are critical in developing forest conservation and reforestation strategies.

In order to understand the changes in genetic diversity, inbreeding, patterns of spatial genetic structure and pollen dispersal, we used ten microsatellite loci to compare an undisturbed, dense *A. angustifolia* population located within an Araucaria Forest fragment (forest cluster) with seeds from a younger population located in pastureland adjacent to the forest cluster (open forest). The analyzed forest cluster is located at EMBRAPA's Research Station in Caçador (ERSC), Santa Catarina State, Brazil, one of the largest areas with Araucaria Forest in a stage of pristine conservation. The forest cluster has an area of seven ha and all 295 adult *A. angustifolia* trees within the cluster were sampled. Seeds were collected from 13 seeds trees in the forest cluster and eight seed trees from the neighboring open forest area. Our analysis aims to answer the following questions: (I) Are there differences in the levels of genetic diversity between established trees and seeds? (II) Is there SGS in the forest cluster and does it affect the genetic diversity and inbreeding levels of open-pollinated seeds? (III) Are there differences in the pollen dispersal distances between the forest cluster and open forest?

3 MATERIAL AND METHODS

3.1 STUDY AREA AND SAMPLING STRATEGY

The EMBRAPA Research Station in Caçador (ERSC) covers an area of 1,157 ha and it is located in the municipality of Caçador, Santa Catarina State, Brazil (25°32'29.64" S and 50°33'44.58" W). The altitude varies from 920 to 1,075 m a.s.l. and the main soil types are the latosol and nitosol. Average precipitation is around 1,600 mm in a subtropical mesothermic humid climate (Cfb, *Koeppen* classification) with an average temperature of 16.6° C; the warmest month is February and the coldest is July, with severe frosts in the winter months. The landscape is dominated by small-scale farms (agriculture and pasture) as well as much larger commercial pine plantations managed by forest companies. Native forests are mostly restricted to

small fragments of Ombrophilous Mixed Forest in varying stages of secondary succession, with the exception of the ERSC, where Araucaria Forest can be found in primary stages of succession (MATTOS *et al*., 2010; LACERDA *et al*., 2012).

The ERSC is mostly covered by forest (94%) and has a history of low intensity timber exploitation that occurred until about 70 years ago when it became government property in 1948 and was designated a site for *in situ* Araucaria Forest conservation. However, despite being recognized as one of most well preserved sites of Araucaria Forest, selective logging continued at low intensities from the mid twentieth-century until the 1990s. In ERSC, *A*. *angustifolia* dominates the canopy in clusters and the species is associated with other valuable hardwood trees such as *Ocotea porosa* and *Cedrela fissilis* and the economically important non-timber species *Ilex paraguariensis* (Sousa et al. 2004). In areas previously subjected to more intense harvesting, the occurrence of abundant populations of various bamboo species (*Merostachys* spp. and *Chusquea* spp.) is common which is related to low levels of forest regeneration (LACERDA *et al*., 2012). Specifically in relation to *A*. *angustifolia*, bamboos and other factors (such as seed and seedling predation) seem to be affecting the species' capacity to regenerate as saplings are rare in the area.

To carry out this study, we selected a seven ha dense cluster of *A. angustifolia* inside the ERSC. All *A. angustifolia* trees in the study area had their d.b.h., geographic coordinates and sex identified; for DNA analysis, vascular cambium samples were also taken. In total, all 295 adult trees within the forest cluster were identified and sampled together with seeds from 13 randomly selected seed trees. Seeds from eight trees located in the adjacent open forest area were also collected. From each seed tree, we genotyped 28 seeds from a single cone, with the exception of one seed tree located in the open forest, from which only 14 seeds were genotyped. We sampled seeds from only one cone per seed tree due to the low rate of cone production per reproductive tree and the costs associated with sampling cones in the field; it was deemed preferable to collect samples from a greater number of seed trees rather than from several cones from a small number of trees. An implication of this sampling strategy is that it may lead to an overestimation of instances of correlated mating.

The adult trees did not show significant deviation from the 1:1 sex ratio (143 males and 152 females) and their diameter distribution varied from 20 cm to 176 cm DBH with an average of 76 cm. Based on a dendrochronology study by Mattos *et al*.

(2010) carried out at the ERSC, the estimated age of sampled *A. angustifolia* trees ranged from 40 to 332 years.

3.2 MICROSATELLITE ANALYSIS

The isolation of DNA from *A. angustifolia* seeds followed the protocol described by Mazza and Bittencourt (2000). The same protocol was applied to the vascular cambium tissue from adult trees, with minor modifications, eliminating the proteinase K and CTAB 10% (NaCl 1.4 M) solutions. Quantification was performed comparing 5 µL of the DNA from each sample with 5 µL of Phage Lambda DNA size marker with concentrations of 20, 50 and 100 ng in 2% agarose gel. After quantification, each sample was diluted with autoclaved milli-Q water to a final concentration of 10 ng/µL to be used in the analyses. Of the 20 pairs of microsatellite markers available for *A. angustifolia*, eight were selected to be amplified in three multiplex systems, as two of these primer pairs were able to amplify unspecific regions (TABLE 1- supplementary material).

The PCR reactions were performed using the Qiagen Multiplex PCR Master Mix in a final volume of 10 µL, containing 5 µL of PCR Master Mix (2x), 1 µL of primer pairs (2 mM each primer), 2 µL of genomic DNA (10 ng/µL), and 1 µL of Q-Solution and Milli-Q water. The PCR program used in the thermocycler involved: (1) an initial step at 95º C for 15 min for DNA denaturation and Taq DNA polymerase activation; (2) 35 cycles of amplification in three stages (94º C for 30 s, annealing temperature for 90 s, and extension at 72º C for 60 s); and (3) a final extension at 72 ºC for 10 min. After amplification, 10 µL of Milli-Q water was added to each sample and refrigerated at 4 ºC until genotyping. For genotyping, we used a solution of 10 µL containing 2 µL of the amplified fragment solution from each sample, 0.125 µL of sequencer standard ROX GS 500 or LIZ GS 600, and the remaining volume with Hi-Di formamide. Polymorphism was detected by labeling SSR primer pairs marked with fluorescent dyes in triplex or duplex combinations, followed by capillary electrophoresis to detect fragments in a 3500xL ABI Genetic Analyzer automated sequencer (Applied Biosystems). The size of amplified fragments (alleles) was determined using the GeneMapper v.4.1 software (Applied Biosystems) and the

values referring to the size of the alleles were exported to a spreadsheet for statistical analysis.

3.3 ANALYSIS OF GENETIC DIVERSITY AND FIXATION INDEX

The genetic diversity of the adults and seeds both inside and outside of the forest cluster were estimated by the number of alleles (A), observed heterozygosity (H_o) and expected heterozygosity at Hardy-Weinberg equilibrium (H_e) for each locus and as an average across all loci. Because the sample size for the adults and seeds was different, we also calculated the allelic richness (*R*) using a rarefaction method (EL MOUSADIK; PETIT, 1996). The level of inbreeding in the adults and seeds was estimated using the fixation index (*F*). For seeds, the intra-individual fixation index was calculated using reference allele frequencies estimated from the adult trees, using the SPAGeDI 1.3 program (HARDY; VEKEMANS, 2002). The significance of the *F* values was calculated using the permutations of the alleles among individuals (1,000) and a sequential Bonferroni correction for multiple comparisons (95%, α= 0.05). Except for the analysis of the intra-individual fixation index in seeds, all other analyses were run using the FSTAT program, version 2.9.3.2 (GOUDET, 1995). The t-test was used to determine if there are significant differences among the samples for *A, R,* H_o, H_e and *F*.

3.4 ANALYSIS OF INTRAPOPULATIONAL SPATIAL GENETIC STRUCTURE

The intrapopulation spatial genetic structure (SGS) was only investigated for the forest cluster where all trees were sampled, using the average coancestry coefficient (θ_{xy}) between pairs of adult trees, as described by Loiselle *et al*. (1995) using the SPAGeDI 1.3 program (HARDY; VEKEMANS, 2002). To visualise the SGS, θ_{xy} values were averaged in 15 m distance classes (0-150 m), which were then plotted against the distances classes. To test whether there was significant deviation

from a random structure, the 95% confidence interval (CI) was calculated for each observed value and each distance class based on 1,000 permutations of individuals among distance classes. To compare the SGS between the adult *A. angustifolia* trees, the statistic S_p (VEKEMANS; HARDY, 2004) was calculated as $S_p = -b_k / (1 - \theta_1)$, where θ_1 is the average coancestry coefficient calculated between all the pairwise individuals within the first distance class (0-15 m), and b_k is the slope of the regression of coancestry coefficient on the logarithm of spatial distance (0-150 m). To test for SGS, the spatial position for each individual was permuted (1,000 times) to obtain the frequency distribution of b_k under the null hypothesis that θ_1 and ln (d_{xy}) are not correlated.

3.5 ANALYSIS OF HISTORICAL GENE DISPERSAL FROM SGS

The historical gene dispersal for adults and seedlings was estimated from the SGS with the assumption that the observed SGS represents an equilibrium isolation-by-distance pattern (HARDY *et al*., 2006). The neighbourhood size (*Nb*) was estimated as $Nb = -(1-\theta_1)/b_k$ (VEKEMANS; HARDY, 2004), where b_k is the regression slope within the distance class of $\sigma_g < d_{ij} < 20\sigma_g$. This estimate of *Nb* is dependent on the assumed value for effective density, D_e (HARDY *et al*., 2006). Thus, D_e was estimated as $D_e = D(N_e / N)$, where the effective density is the ratio of the effective size to the census population size (VEKEMANS; HARDY, 2004). Based on other plant studies (HARDY *et al*., 2006), we used *D*/10 and *D*/2 as minimum and maximum estimates of D_e. We also tested $D_e = D$. In fixing D_e, the lower and upper boundaries for the 95% confidence interval (CI) of *Nb* were respectively estimated as $Nb_{(lower)} = (\theta_1 - 1)/(b_k - 2SE_b)$ and $Nb_{(upper)} = (\theta_1 - 1)/(b_k + 2SE_b)$, where SE_b is the standard error of b_k, calculated by jackknifing data across loci (HARDY *et al*., 2006). The 95% CI of σ_g was estimated as $\sigma_g = \sqrt{N_b / 4\pi D_e \hat{r} D_{ep}}$ using the lower and upper *Nb* boundaries (HARDY *et al*., 2006). According Hardy *et al*. (2006), when $b_k < SE_b$, the upper boundary must be reported as infinite (∞). We also assumed the effective density as the actual density ($D_e = D$).

3.6 ANALYSIS OF VARIANCE EFFECTIVE SIZE

The variance effective size of the reproductive population was calculated as: $\hat{N}_e = 0.5/\Theta$ (COCKERHAM, 1969); where Θ is the average group coancestry. For sexed adults, the average group coancestry was estimated as: $\hat{\Theta} = \sum_{x=1}^{n_f}\sum_{y=1}^{n_f}\hat{\theta}_f / 4n_f^2 + \sum_{x=1}^{n_m}\sum^{n_m}\hat{\theta}_m / 4n_m^2 + \sum_{x=1}^{n_f}\sum_{y=1}^{n_m}\hat{\theta}_{fm} / 2n_f n_m$ (LINDGREN; MULLIN, 1998); where θ_f, θ_m, and θ_{fm} are the coancestry coefficient between females, males, and females and males together, respectively; and n_f and n_m are the number of female (152) and male trees (143). The pairwise coancestry coefficients were estimated using the J. Nason method described by Loiselle *et al*. (1995) in the SPAGeDI 1.3 program (HARDY; VEKEMANS, 2002).

3.7 PATERNITY ANALYSIS

The combined non-exclusion probability of parent pair and genetic identity were calculated using the CERVUS 3.0 program (MARSHALL *et al*., 1998; KALINOWSKI *et al*., 2007). The cryptic gene flow, or the probability of assigning a candidate mother or father inside the population when the true father is outside of the population, was calculated based on Dow and Ashley (1996). Parentage analysis was conducted by maximum-likelihood paternity assignment (MEAGHER, 1986) based on the multilocus genotypes of the 364 seeds collected inside the forest cluster, 210 seeds collected from the open forest, their respective maternal genotypes and all 143 adult male trees present in the cluster as putative pollen parent.

The most likely parent pairs were determined by the Δ statistic (MARSHALL *et al*., 1998) using the reference allele frequencies calculated in the adult population (MEAGHER; THOMPSON, 1987). Significance for Δ was determined using paternity tests simulated by the software (critical Δ), using a confidence level of 80%, a genotyping error ratio of 0.01 and 10,000 repetitions, as suggested by Marshall et al.

(1998). As the species is wind-pollinated and the reproductive population may be higher than the sampled 143 male adult trees in the cluster, in the simulations we arbitrarily assumed 200 male trees as putative pollen parent candidates. The calculation of critical Δ values was based on the assumption that 70% of the candidates sampled were located within the plot. If a father candidate or parent pair had a Δ value higher than the critical Δ value calculated by simulations, it was considered to be the true parent or true parent pair. The pollen immigration rate (*m*) was calculated as the proportion of seeds that had no pollen parents ($n_{immigrant}$) inside the population relative to the total number of sampled seeds (n_{total}): $m = n_{immigrant} / n_{total}$ (BURCZYK *et al*., 1996). As all sampled individuals had a known spatial position, the distance of pollen dispersal was based on the position of the putative seed tree in relation to the putative pollen donor. To investigate whether reproductive success was a function of the distance between trees, we compared the frequency distribution of pollen dispersal with the frequency distribution of distance between all adult *A. angustifolia* trees using the Kolmogorov-Smirnov test (SOKAL; ROHLF, 1995).

The effective pollination neighbour area (A_{ep}) was calculated by taking a circular area around a seed tree, $\hat{A}_{ep} = 2\pi\hat{\sigma}^2$ (LEVIN, 1988), where σ_p^2 is the axial pollen dispersal variance. It is important to note that that the parameter A_{ep} corresponds to the circular area in which 63% of pollen donors that crossed with a seed tree are expected to be located (LEVIN, 1988). The circular pollination radius was estimated as: $\hat{r}_{ep} = \sqrt{\hat{A}_{ep} / 3.14159}$ (AUSTERLITZ; SMOUSE, 2001).

4 RESULTS

4.1 GENETIC DIVERSITY AND FIXATION INDEX

Of the eight SSR primers used in the genetics analysis, two of them amplified unspecific regions that segregated in a Mendelian pattern (Aang28b and CRCAc1b); as such, an overall total of ten microsatellite loci were used in this study (Table 1). Of

the 869 DNA samples, the number of alleles (k) from the ten analysed loci ranged from 4 to 16, producing a total of 88 different alleles and an average of 8.8 alleles per locus.

Comparing adults to seeds (TABLE 1), we found 81 alleles in the sampled adults, 84 in seeds from the forest cluster, and 78 in seeds from the open forest. Only two alleles were private to the adult forest population; one was found in a male tree and the other in a female tree. Three alleles were found solely in seeds and male trees and not in female trees. Another seven alleles were absent from the sampled adult population, including five found in seeds from the open forest, and two in seeds collected from the forest cluster.

The allelic richness (R) ranged from 4.0 to 15.1 for adults in the forest cluster (average of 7.4), from 4.0 to 15.8 for seeds in the forest cluster (average of 8.2), and from 4.0 to 13.9 for seeds from the open forest (average of 7.5). The observed heterozygosity (H_o) and expected heterozygosity (H_e) showed slight differences, with the H_o values always being higher than H_e for all loci, resulting in negative fixation index (F) values across all ten loci and no evidence of null alleles.

For adult trees of the forest cluster, the H_o ranged from 0.71 to 1.00 (average of 0.91). For seeds inside the forest cluster, H_o varied from 0.60 to 0.98 (average of 0.85), and for seeds from the open forest it ranged from 0.66 to 0.98 (average of 0.85). The expected heterozygosity (H_e) of adult trees ranged from 0.58 to 0.78 (average of 0.70), for seeds from inside the forest cluster H_e ranged from 0.55 to 0.88 (average of 0.74), and for seeds from the open forest it ranged from 0.55 to 0.84 (average of 0.68).

For adults in the forest cluster, all loci and the average across all loci produced significantly lower than zero fixation index (F), with an average of -0.31. For seeds collected from the forest cluster, the F values were significantly lower than zero for seven loci and for the average across all loci (-0.15). For seeds collected from the open forest, the F values were significantly lower than zero for nine loci and for the average across all loci (-0.25). The t-test (TABLE 2) indicates that F values in adult trees in the forest cluster were significantly lower than their seeds (P = 0.001), but not in relation to seeds from the open forest (P = 0.154). Moreover, the F values of seeds from the open forest were also significantly lower than seeds from the forest cluster (P = 0.046).

TABLE 1 - GENETIC DIVERSITY AND FIXATION INDEXES FOR ALL ADULT TREES AND SEEDS FROM WITHIN AND OUTSIDE THE *Araucaria angustifolia* FOREST CLUSTER.

Locus	Adults								Seeds of Forest Cluster						Seeds of Open Area					
	n	k	R	H_o	H_e	F	P_{2_parent}	$P_{identity}$	n	k	R	H_o	H_e	F	n	k	R	H_o	H_e	F
AG45	295	6	5.3	0.93	0.67	-0.39*	0.547	0.257	364	6	6.0	0.78	0.67	-0.16*	210	5	4.7	0.66	0.55	-0.20*
AS190	295	9	7.7	0.97	0.74	-0.32*	0.258	0.085	364	9	8.9	0.92	0.75	-0.23*	210	8	8.0	0.91	0.67	-0.36*
CRCAC1a	295	7	6.6	0.71	0.61	-0.17*	0.428	0.168	364	7	7.0	0.60	0.55	-0.08	210	7	7.0	0.68	0.56	-0.22*
CRCAC1b	295	7	6.4	0.77	0.58	-0.32*	0.311	0.113	364	10	9.4	0.81	0.79	-0.03	210	9	7.7	0.82	0.65	-0.27*
AG20	295	10	9.3	0.89	0.76	-0.17*	0.440	0.196	364	10	9.6	0.87	0.76	-0.14*	210	9	8.0	0.81	0.63	-0.28*
AG56	295	8	7.1	1.00	0.78	-0.29*	0.267	0.094	364	8	8.0	0.98	0.82	-0.20*	210	8	7.9	0.97	0.72	-0.35*
Aang01	292	16	15.1	0.96	0.75	-0.28*	0.324	0.113	364	16	15.8	0.96	0.88	-0.09*	210	14	13.9	0.93	0.84	-0.11*
Aang14	292	8	7.1	0.92	0.62	-0.47*	0.373	0.137	364	8	8.0	0.80	0.74	-0.09*	210	8	7.9	0.82	0.77	-0.06
Aang28a	292	6	5.7	0.99	0.74	-0.34*	0.221	0.083	364	6	5.8	0.82	0.76	-0.07	210	6	6.0	0.90	0.72	-0.26*
Aang28b	292	4	4.0	1.00	0.71	-0.41*	0.463	0.203	364	4	4.0	0.98	0.70	-0.39*	210	4	4.0	0.98	0.71	-0.38*
Mean	--	8.1	7.4	0.91	0.70	-0.31*	--	--	--	8.40	8.2	0.85	0.74	-0.15*	--	7.80	7.5	0.85	0.68	-0.25*
SD	--	3.2	3.1	0.10	0.07	0.10	--	--	--	3.27	3.2	0.12	0.09	0.10	--	2.74	2.7	0.11	0.09	0.11
Total	--	81	--	--	--	--	2.74^{-4}	1.99^{-9}	--	84	--	--	--	--	--	78	--	--	--	--

n is the sample size; k is the number of alleles; R is the allelic richness for 143 individuals; H_o is the observed heterozygosity; H_e is the expected heterozygosity; F is the fixation index; P_{2_parent} is the combined non-excluding probability of the parent pair; and $P_{indentity}$ is the combined non-exclusion probability of identity. * P<0.05.
SOURCE: The author.

TABLE 2 - PROBABILITIES OF T-TEST (P VALUES) FOR DIFFERENCES IN THE GENETIC PARAMETERS AMONG ADULTS VS SEEDS OF THE FOREST CLUSTER (AD VS SEEDS CLUSTER), ADULTS VS SEEDS IN THE OPEN FOREST (AD VS SEEDS OPEN AREA) AND SEEDS OF FOREST CLUSTER VS SEEDS FROM OPEN FOREST (SEEDS CLUSTER VS SEEDS OPEN AREA) IN *Araucaria angustifolia*.

Parameter	Ad vs Seeds Cluster	Ad vs Seeds Open	Seeds Cluster vs Seeds Open
A	0.893	0.825	0.662
R	0.564	0.951	0.581
H_o	0.218	0.180	0.940
H_e	0.219	0.703	0.152
F	0.001	0.154	0.046

A is the average number of alleles; R is the allelic richness; H_o is the observed heterozygosity; H_e is the expected heterozygosity; F is the fixation index.
SOURCE: The author.

4.2 INTRAPOPULATIONAL SPATIAL GENETIC STRUCTURE AND HISTORICAL GENE DISPERSAL

The correlogram showed a typical pattern of isolation by distance, with the average pairwise coancestry coefficient being significant up to 90 m, suggesting that near-neighbour individuals are related (FIGURE 1).

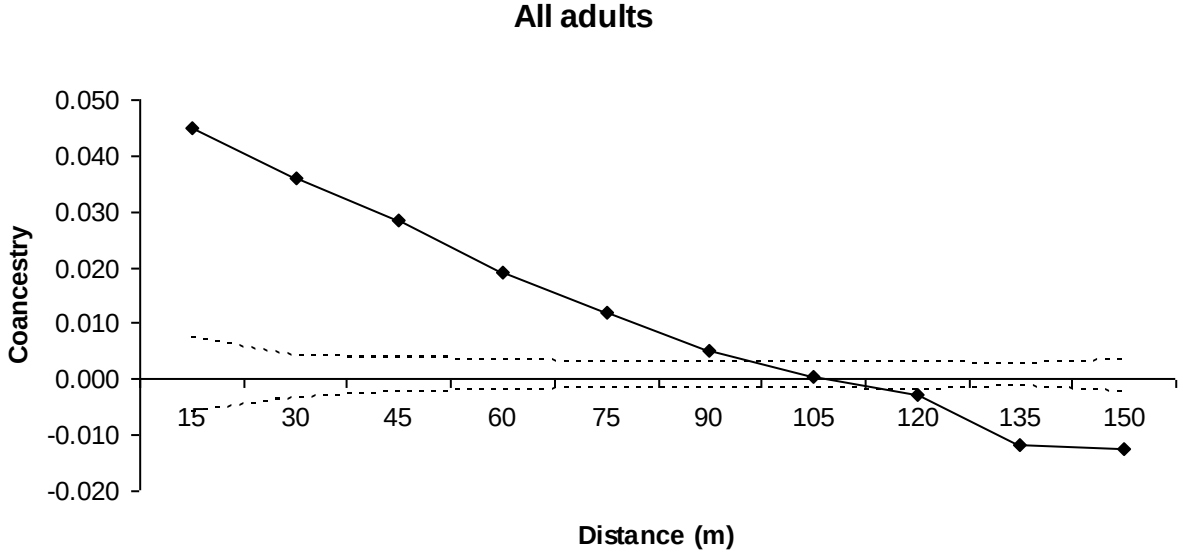

FIGURE 1 - CORRELOGRAMS OF AVERAGE COANCESTRY COEFFICIENTS (Θ_{XY}) FOR ALL *Araucaria angustifolia* ADULTS, AND FOR MALES AND FEMALES FOR TEN

DISTANCE CLASSES WITH INTERVALS OF 15 M. THE SOLID LINE REPRESENTS THE AVERAGE Θ_{XY} VALUE. THE DASHED LINES REPRESENT THE 95% (TWO-TAILED) CONFIDENCE INTERVAL OF THE AVERAGE Θ_{XY} DISTRIBUTION CALCULATED BY 1000 PERMUTATIONS OF SPATIAL DISTANCE BETWEEN PAIRS OF ADULTS AND SEEDLINGS.

SOURCE: The author.

Beyond the 90 m distance, the average coancestry coefficient drops from not significantly different from zero to significantly lower than zero. The regression slope (b_k) of pairwise coancestry coefficient on the logarithm of spatial distance (0-150 m) was significantly negative, confirming the occurrence of isolation by distance (TABLE 3). The intensity of the SGS measured by S_p statistic was 0.0081. Assuming $D_e = D$ and $D_e = D/2$, the estimates of historical gene dispersal distance (σ_g), based on the restricted b_k (0-150 m), produced a dispersal of 28 m and 40 m, respectively, with an effective neighbour size (*Nb*) of 38 and 40 individuals. Assuming $D_e = D/10$, σ_g and *Nb* did not converge.

TABLE 3 - ESTIMATES OF INTRAPOPULATIONAL SPATIAL GENETIC STRUCTURE PARAMETERS FOR ALL ADULTS OF THE STUDIED *Araucaria angustifolia* POPULATION.

SGS parameters	All adults (n = 295)
θ_1	0.0448***
b_k (SE) (0-150 m)	-0.0077 (0.0025)
S_p (SE)	0.0081
Nb (SE)	38 (11)
σ_g (SE (m)($D_e = D$)	28 (4)
Nb (SE)	40 (14)
σ_g (SE (m)($D_e = D/2$)	40 (6)
Nb (SE)	NC
σ_g (SE (m)($D_e = D/10$)	NC

θ_1 is the coancestry coefficient in the first distance class (0-15 m); b_k is the regression slope of the coancestry coefficient value on the logarithm of spatial distance between individuals located within the distance between 0 to 150 m; S_p is the statistic that measures the extension of the spatial genetic structure in the first distance class (0-15 m); *Nb* is the neighbourhood size; σ_g is the historical gene dispersal distance for two estimated effective densities ($D_e = D/2$, $D_e = D/10$ and $D_e = D$); NC= not converged; *** $P<0.001$. Male and females, $\theta_1 = 0.0470$.

SOURCE: The author.

4.3 EFFECTIVE POPULATION SIZE

The average coancestry coefficient among groups of females, males, and males and females together, was 0.00193, 0.00120 and 0.00596, respectively, indicating that in random mating the expected biparental inbreeding in offspring is very low (total of 0.00910, TABLE 4). The estimated variance effective size of the reproductive population indicates that of the 295 adult trees 55 individuals are neither related nor inbred ($N_e / N = 0.19$).

TABLE 4 - RESULTS OF COANCESTRY GROUPS AND EFFECTIVE POPULATION SIZE FOR ADULT *Araucaria angustifolia* POPULATIONS.

Parameters	This paper	Sant'Anna *et al*. (2013)	Bittencourt and Sebbenn (2007)
Population site	ERSC at Caçador, SC State	ERSC at Caçador, SC State	Private Property at Mangueirinha – PR State
Population history	Minimal logging +50 years ago	Medium intensity logged < 50 years ago	Isolated fragment minimally logged <50 years ago
Evaluated area (ha)	7	7.2	5.4
Total number of trees: N	295	215	220
Density (trees/ha)	42.1	40.3	40.7
Total number of female trees: N_f	152	100	99
Total number of male trees: N_m	143	115	121
Group coancestry among females: θ_f	0.00193	0.00073	0.00023
Group coancestry among male: θ_m	0.00120	-0.00072	0.00103
Group coancestry among females and male: θ_{fm}	0.00596	0.00043	0.00034
Group coancestry: Θ	0.00910	0.00077	0.00161
Effective population size: N_e	55	162	121
Relation N_e / N	0.19	0.75	0.53

SOURCE: The author.

4.4 PATERNITY ANALYSIS

The combined non-exclusion probability for the parent pair across the ten loci was very low (P_{2_Parent} = 2.74^{-4}, TABLE 1). Thus, the probability of cryptic pollen flow was low [0.004= 1-(1-0.0000274)^{143}] and polymorphism of these loci are suitable for parentage analysis. The combined non-exclusion probability of identity was also extremely low ($P_{identity}$ = 1.99^{-9}, TABLE 1), indicating that all multilocus genotypes and individuals are unique, which is optimal for paternity analysis. Among the 364 seeds sampled inside forest cluster, a pollen donor was found for 268 seeds (74 %). Of the 210 seeds sampled from the open forest, a father was found for 169 (80 %), indicating substantial pollen immigration for seed trees within both the forest cluster (26 %) and the open forest (20 %). Based on the results from the seeds with assigned paternity, the estimated number of pollen donors pollinating each seed tree varied from 12 to 20 inside the forest cluster, while for seed trees from the open forest it ranged from 9 to 20 pollen donors (TABLE 5).

TABLE 5 - EFFECTIVE PARAMETERS OF POLLEN DISPERSAL FOR *Araucaria angustifolia* POPULATIONS WITHIN AND OUTSIDE THE FOREST CLUSTER.

	Gene flow (absolute number)			Dispersal distance					N_{ep}	
Sample	n	Within	Outside	Mean $\pm$ SD (m)	Median (m)	Min/Max (m)	A_{ep} (ha)	r_{ep} (m)	Mean	Min/Max
Forest Cluster	364	74% (268)	26% (98)	105 $\pm$ 53	99	7/263	1.76	75	15	12/20
Open Forest	210	80% (169)	20% (43)	298 $\pm$ 57	288	178/458	2.07	81	15	9/20

n is the sample size; SD is the standard deviation of pollen dispersal distance; Min/Max is the minimum and maximum values of progeny parameters, respectively; A_{ep} is the pollination neighbour area; r_{ep} is the radius of effective pollination area; N_{ep} is the number effective of pollen donors.

SOURCE: The author.

The pollen dispersal distance inside the forest cluster ranged from 7 to 263 m, with an average of 105 m and median of 99 m. The results from the open forest located outside the fragment showed an estimated pollen dispersal that was substantially higher, ranging from 178 to 458 m, with an average of 298 m and a median of 288 m (TABLE 5).

Inside the forest cluster, approximately 81% of the assigned pollen travelled less than 150 m and 95% travelled less than 200 m. In contrast, 56% of pollen assigned to seed trees from the open forest came from male trees located up to 300 m from the seed trees, and 97% up to 400 m. However, the average effective pollination neighbour area (A_{ep}) was similar in the forest cluster (11 ha) and open forest (13 ha), resulting in an average radius of pollen dispersal (r_{ep}) of 75 and 81 m, respectively.

Comparing the frequency curve of effective pollen dispersal with the frequency curve measured among all reproductive trees using the Kolmogorov-Smirnov test, significant differences were detected in both the forest cluster (D = 0.087, $P = 0.038$) and open forest (D = 0.567, $P < 0.001$), suggesting a non-random distribution of pollination distances in both sample areas (FIGURE 2). There was a significant association between the number of seeds fertilized by pollen donors and the distance between the paternal and maternal trees in the forest cluster (linear regression: R^2 = 0.35, df = 9, P< 0.01; exponential regression: R^2 = 0.56, df = 9, P< 0.01) and open forest (linear regression: R^2 = 0.17, df = 10, P< 0.05; exponential regression: R^2 = 0.28, df = 10, P< 0.01), indicating that likelihood of mating is related to distance among individuals.

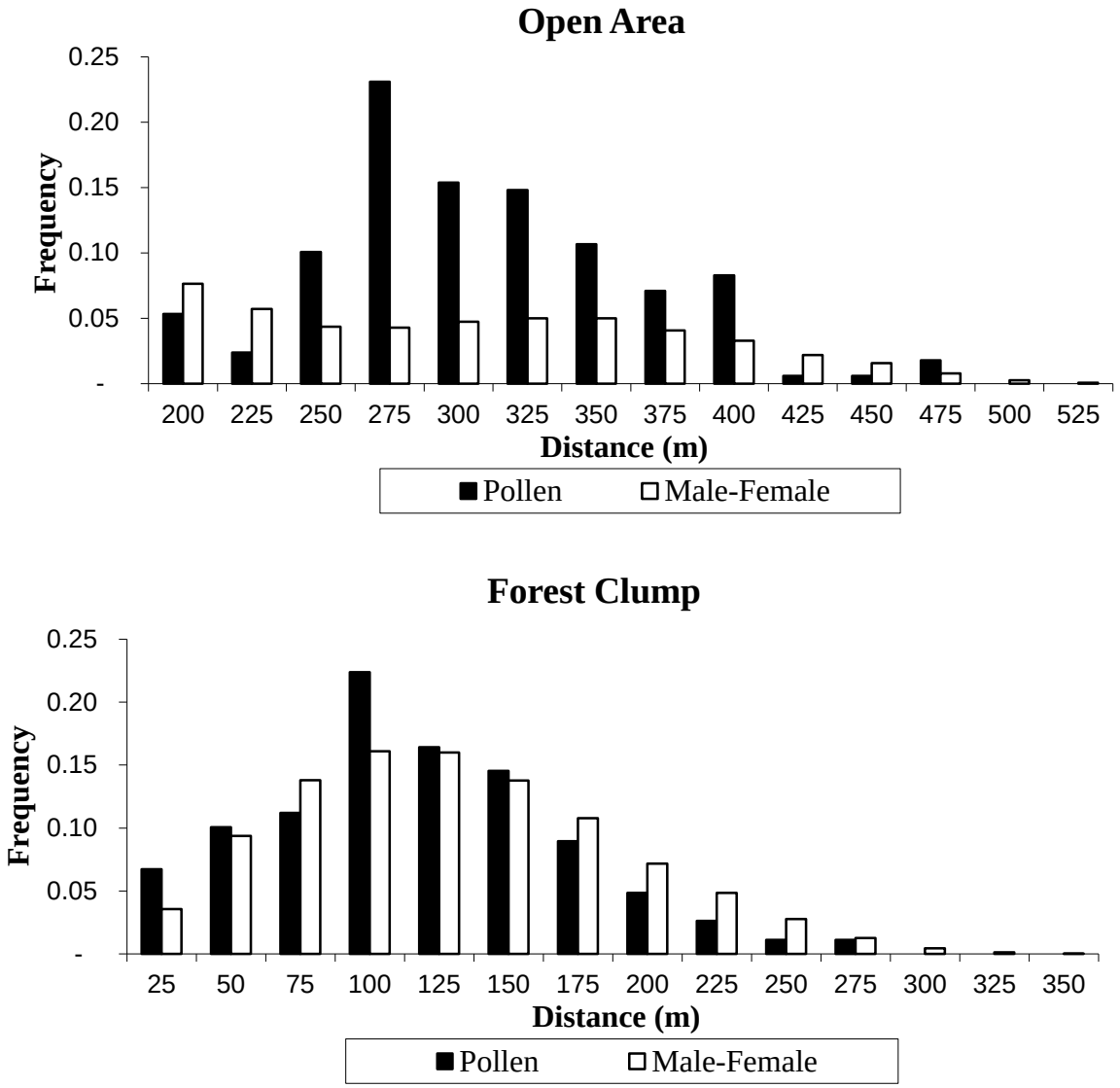

FIGURE 2 - POLLEN DISPERSAL DISTANCE DETERMINED BY PATERNITY ANALYSIS IN SEEDS AND DISTANCE AMONG MALE AND FEMALES TREES IN THE FOREST CLUSTER AND OPEN FOREST.
SOURCE: The author.

5 DISCUSSION

5.1 GENETIC DIVERSITY

Private alleles were found in both the adults and seeds sampled. The private alleles found in adult *A. angustifolia* trees were present in only one male and another

in one female. These alleles would likely have been observed in the seed sample if the sampling effort had been more extensive. Considering that *A. angustifolia* cones contain up to 150 viable seeds and we sampled up to 28 seeds from each cone, it is possible that the private alleles found in the one male and one female may occur in seeds that were not sampled. However, the seven private alleles found in the total seed sample indicate pollen immigration into a site where there is already a large reproductive population. Of the seven private alleles, five were observed in seeds sampled from trees located in the open forest area, whereas two private alleles are from seeds collected within the forest cluster. Both private alleles found in seeds from the forest cluster are definitively different from the alleles found in the adult population (forest cluster) as a census was carried out as part of this study. These results are consistent with those found by Bittencourt and Sebbenn (2007, 2009) who reported fewer external alleles in mature forests than in seed trees located in open areas or secondary forests.

Our results indicate that the forest cluster and open forest share the same pollen gene pool. The number of alleles (k), allelic richness (R), observed (H_o) and expected (H_e) heterozygosity between adults and seeds from the forest cluster and seeds from the open forest were not significantly different (TABLE 2). Comparing the observed heterozygosity from adult trees (0.91) with other studies (TABLE 2 - supplementary material), we can see that our estimate of H_o is higher than that reported in most studies (maximum of 0.81).

It is very difficult to compare genetic diversity parameters among populations of the same species with different sample sizes, both in terms of number of individuals and number of loci of the same genetic markers; additionally, the fact that markers are usually produced by different companies and data is analysed using different equipment contributes to the difficulty. However, in comparing our results for H_o with another study conducted in the ERSC based on nine microsatellite loci (H_o = 0.58 - SANT'ANNA *et al*., 2013), our conclusion is consistent: the forest cluster population presents high levels of observed heterozygosity.

5.2 BIPARENTAL INBREEDING

The average group coancestry coefficient among males and females indicates that in random mating the expected biparental inbreeding in offspring is very low (0.00910, TABLE 4). The average values of fixation index were negative for all samples (TABLE 1), indicating an absence of inbreeding. However, the average F value in adults of the forest cluster was significantly lower than seeds of the forest cluster, suggesting selection for heterozygous individuals between seed to adult stages. Furthermore, inside the forest cluster we also found that the median pollen dispersal distance (99 m) was similar to the significant distance found for SGS (90 m, FIGURE 1). This suggests a high probability of mating among related individuals, despite the fact that we did not detect inbreeding in the seeds. The lack of inbreeding found in the seeds suggests the existence of genetic mechanisms that inhibit mating among related individuals. It is important to note that the estimate of F is based on samples that are a likely mix of inbred and outbred individuals. As the proportion of outbred individuals was probably higher than inbred, the values for F were negative.

5.3 SGS AND COANCESTRY OF ADULT TREES

The correlograms of average coancestry coefficients (FIGURE 1) showed a typical pattern of isolation by distance. The estimated coancestry coefficient in the first distance class (0-15 m) in all adults (θ_1= 0.0448) is in agreement with Hardy *et al*. (2006), who describe that the magnitude of spatial genetic structure is relatively low in high density tree species. These results also agree with a previous study of *A. angustifolia* realized inside the ERSC, which found coancestry levels lower than 0.04 for both adult and juvenile trees within the first 20 m distance class (SANT'ANNA *et al*., 2013). Similarly, Stefenon *et al*. (2007), using both SSR and AFLP markers in six *A. angustifolia* populations over a large distribution area in Brazil, found a coancestry coefficient in the first distance class (1-10 m) ranging from -0.009 to 0.039 for SSR analysis, and from -0.009 to 0.116 for AFLP. In contrast, from an undisturbed *A. angustifolia* forest fragment, Bittencourt and Sebbenn (2007) found coancestry levels

that were slightly higher than those expected for half-sibs in adults (0.125-0.19), first cousins in juveniles (0.08-0.18), and full-sibs in seedlings (0.15-0.25) in the first 25 m distance class. In a protected continuous forest in an indigenous reserve, Bittencourt and Sebbenn (2008) also found higher coancestry levels in seedlings (0.169-0.179) than in the adult population (0.078).

The range of significant SGS found in the studied forest cluster (up to 90 m) is greater than that reported for other *A. angustifolia* populations. Significant SGS has been detected in the populations of the species between 60 to 80 m (MANTOVANI *et al*., 2006; BITTENCOURT; SEBBENN, 2007, 2008; STEFENON *et al*., 2008; SANT'ANNA *et al*., 2013). The intensity of SGS measured by Sp statistic (0.0081) was also higher than that reported by Sant'Anna *et al*. (2013; Sp = 0.0026), indicating that the SGS in the forest cluster is strong. Although some of the cited studies were conducted in the ERSC, they also include clusters of *A. angustifolia* from other regions which may not have been subjected to the same levels of logging. Considering what we known about the forest structure, diversity of species and historical information, the forest cluster analysed in this study was likely logged before the area became government property in 1948, with selective harvesting targeted at very large individuals.

The estimated historic gene dispersal in terms of neighbourhood size (*Nb*) converged to the nearest 40 trees (TABLE 2) and a distance of historic gene dispersal (σ_g) ranged from 28 m to 40 m. This value is similar to the observed population density per hectare in the area (42 trees). The *Nb* and σ_g values are substantially lower than those reported for adult trees in other *A. angustifolia* populations from the ERSC, and these differences may be attributed to historical logging events. The forest cluster analysed in this study, together with the area evaluated by Bittencourt and Sebbenn (2008) in an indigenous reserve in Parana State were subjected to low levels of disturbance. This can explain the absence of regeneration. On the other hand, the plot studied by Sant'Anna *et al*. (2013) has been logged more recently and/or more intensely (*Nb*= 391 and σ_g = 140 for D_e = *D*/2) and juvenile individuals were identified within their study area. The occurrence of *A. angustifolia* in disturbed forests seems consistent with the species ecological characteristics (discussed further below).

5.4 EFFECTIVE POPULATION SIZE

Our estimate of variance effective size (N_e) of the adult population was very low ($N_e / N = 0.19$). The low N_e value is due to the occurrence of related individuals within the forest cluster, which increases the frequency of identical by decent alleles (IBD) among individuals, reducing the N_e. Interestingly, our estimated rate between the effective population size and the sample size ($N_e / N = 0.19$) was lower than that estimated by Sant'Anna *et al*. (2013) in a different part of the same forest, subjected to more recent/higher intensity logging ($N_e / N = 0.75$), and lower than that found by Bittencourt and Sebbenn *et al*. (2007) in a spatially isolated *A. angustifolia* forest fragment ($N_e / N = 0.53$).

5.5 POLLEN DISPERSAL AND CORRELATED MATING

Pollen immigration was detected in both the forest cluster and open forest (minimum of 20%), indicating intensive pollen movement into these sub-populations. Low levels of pollen flow have been reported in previous studies on *A. angustifolia* in fragmented and continuous forests. For example, Sant'Anna *et al*. (2013) studying the realized pollen immigration in juveniles within a 7.2 ha area (also located in the ERSC) found 6% of pollen immigration. In a spatially isolated population in a fragmented landscape, Bittencourt and Sebbenn (2007) found an effective pollen immigration rate of 10% and a realized pollen immigration rate of 3% for seedlings and 7% for juveniles. In contrast, these authors also found an effective pollen immigration rate of 45% in a small and isolated group of 11 *A. angustifolia* trees (BITTENCOURT; SEBBENN, 2007) and an effective pollen immigration of 54% in a 14 ha plot surveyed within a continuous forest (BITTENCOURT; SEBBENN, 2008). In fact, the estimate of pollen immigration in the *A. angustifolia* populations evaluated in this study are lower than that described for other wind-pollinated tree species with relatively low levels of geographic isolation (Table 4 – BITTENCOURT; SEBBENN, 2007), where pollen immigration for wind-pollinated tree species has been reported

ranging from 27% (*Pseudotsuga menziesii* - ADAMS, 1992) to 95% (*Fraxinus excelsior* - BACLES *et al*., 2005).

Our results, along with what was observed by Souza *et al*. (2005), Bittencourt and Sebbenn (2007, 2008, 2009) and Sant'Anna *et al*. (2013), provide us with a more thorough understanding of the ecological gene flow dynamics of *A. angustifolia* trees across various forest conditions. Pollen dispersal distance was higher in seeds sampled from seed trees located in the open forest (mean of 298 m and maximum of 458 m) than from the forest cluster (mean of 105 m and maximum of 263 m). In this case, the greater pollen dispersal distance is related to site characteristics; in open areas the *A. angustifolia* pollen may be dispersed over longer distances in comparison to a dense forest stand, where the influence of tree density is expected to reduce pollen dispersion. This hypothesis is also supported by studies on isolated *A. angustifolia* seed trees occurring in pastures as compared to seeds collected from seed trees within fragmented and continuous forests. Bittencourt and Sebbenn (2007) found pollen dispersal distances up to 2,069 m in a small fragmented population and 1,913 m for isolated seed trees in pastures. The average pollen dispersal distance found within the forest cluster (average 105 m) is similar to results found in the other studies for the same species (TABLE 4), which evaluated Araucaria Forest scenarios with different historical uses (102 m - BITTENCOURT; SEBBENN, 2008; 134 m - SANT'ANNA *et al*., 2013).

Assuming a circular area around the sampled seed trees, within which 63% of the effective pollen donors that mated with the seed tree occurs (LEVIN, 1988), we found that the average effective pollination neighbour area (A_{ep}) was similar in the forest cluster (1.76 ha) and open forest (2.07 ha), resulting in similar average radii of pollen dispersal (r_{ep}; 75 m for forest cluster and 81 m for open forest). Despite the fact that (a) denser forests may restrict pollen dispersal due to the existence of physical barriers, and (b) the linear regression results indicate a tendency of mating among near neighbor individuals (R^2 = 0.35, df= 9, P< 0.01), the higher density of reproductive trees inside the pollination neighborhood area together with genetic mechanisms inhibiting mating among related individuals, contributes to the maintenance of higher levels of diversity in the seeds sampled inside the forest cluster. Our estimate of A_{ep} within the forest cluster is similar to the results reported previously for seeds from other fragmented and continuous populations of the

species (1.7 ha - BITTENCOURT; SEBBENN, 2007, 2008). However, our estimate of A_{ep} in the open forest was higher than that detected for isolated trees in pastures (0.3 ha) by Bittencourt and Sebbenn (2007). These difference between the A_{ep} among isolated trees in similar scenarios is directly related to the differences in the degree of isolation of these areas. The isolated seed trees of our study are closer to forest environments than the seed trees evaluated by Bittencourt and Sebbenn which are isolated by almost 2 km from the nearest Araucaria Forest stand.

6 CONCLUSION

Our results indicate that trees in open areas and near the borders of forest fragments were more likely to receive immigrant pollen and therefore private alleles are more likely to be found among trees located deep inside forest fragments. Thus, strategies aiming at the restoration of *A. angustifolia* populations and conservation of forest fragments should take into account these factors and include ways of guaranteeing regeneration inside forest fragments and/or ensuring that seeds from within forests are being included in seedling production. Conservation strategies could also take advantage of seed trees located near large continuous areas for seed collection. As observed in this study and by Bittencourt and Sebbenn (2007), these trees can receive pollen from a large pollination neighborhood area, producing seeds with high levels of genetic diversity. Additionally, seed collection from open areas is easier, safer and less expensive than collecting from inside dense forests. In open areas, the Araucaria cones are easily accessed without the requirement of special equipment, whereas in dense forests a strenuous and risky specialized climbing operation is necessary.

The studied forest cluster did not show signs of regeneration, indicating the advanced successional stage of the selected case study area (BITTENCOURT; SEBBENN, 2008). The adult *A. angustifolia* population presents high levels of genetic diversity, but substantial and intense SGS and low effective population size. The SGS and low estimated rate of pollen flow can explain the low effective population size in the forest cluster. These factors are also associated with the likely

inbreeding depression in dioecious species, which may explain the high genetic diversity of Araucaria species. These characteristics highlight the importance of this site for *in situ* conservation as it retains high levels of genetic diversity, even considering the low effective population size of the studied forest. However, our results for pollen flow show that the population size is greater than the studied area and the estimated effective population size is obviously an underestimation of the true effective population size. Consequently, seed trees located up to 500 m around forest environments are an interesting source of seeds for tree breeding, *ex situ* conservation and environmental reforestation, particularly because they can provide easily accessible seeds that are likely to retain high levels of genetic diversity from several of the nearest forest populations.

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9 SUPPLEMENTARY MATERIAL

TABLE 1 - MULTIPLEX COMBINATION OF SSR PRIMERS FROM ARAUCARIA SPECIES USED IN THIS STUDY.

SSR primer	Dye	Multiplex	Tm ºC	Repeat motif	Fragment range (pb)	Author	*Araucaria* specie	GenBank accession number
Ag 45	Fam	I	57.8ºC	$(GT)_4$	145 – 180	Salgueiro *et al*. 2005	*A. angustifolia*	AJ749966
AS 190	Ned	I	57.8ºC	$(AT)_8(GT)_{12}$	165 – 190	Robertson *et al*. 2004	*A. subulata*	AY426083
CRCAC1a	Fam	I	57.8ºC	$(GA)_{19}$	180 – 210	Scott *et al*. 2003	*A. cunninghamii*	AF522871
CRCAC1b	Fam	I	57.8ºC	$(GA)_{19}$	75 – 115	Scott *et al*. 2003	*A. cunninghamii*	AF522871
Ag 20	Fam	II	59.3 ºC	$(GA)_{12}$	225 – 260	Salgueiro *et al*. 2005	*A. angustifolia*	AJ749964
Ag 56	Ned	II	59.3 ºC	$(TC)_{11}$	135 – 160	Salgueiro *et al*. 2005	*A. angustifolia*	AJ749967
Aang 01	Hex	III	56.5 ºC	$(CT)_{22}$	200 – 235	Schmidt *et al*. 2007	*A. angustifolia*	AY865575
Aang 14	Fam	III	56.5 ºC	$(GA)_{27}$	200 – 225	Schmidt *et al*. 2007	*A. angustifolia*	AY865583
Aang 28a	Hex	III	56.5 ºC	$(CT)_{11}$	140 – 160	Schmidt *et al*. 2007	*A. angustifolia*	AY865592
Aang 28b	Hex	III	56.5 ºC	$(CT)_{11}$	160 – 175	Schmidt *et al*. 2007	*A. angustifolia*	AY865592

SOURCE: The author.

TABLE 2 - RESULTS OF OBSERVED HETEROZYGOSITY (H_o), FIXATION INDEX (F), INTRAPOPULATION SPATIAL GENETIC STRUCTURE (SGS), Sp STATISTIC, POLLEN IMMIGRATION (m), DISTANCE OF POLLEN DISPERSAL (D) AND EFFECTIVE POLLINATION NEIGHBORHOOD AREA (A_{ep}) IN STUDIES OF *Araucaria angustifolia*. (*, ** AND *** ARE F VALUES SIGNIFICANT AT LEVELS OF 0.90, 0.95 AND 0.99, RESPECTIVELY).

Population	Kind	N locus (marker)	H_o	F	SGS (m)	Sp	m (%)	D mean (maximum) (m)	A_{ep} (ha)	Authors
ADULTS										
Manguerinha	Continue	8 (SSR)	0.55	0.058	75	-	-	-	-	Bittencourt and Sebbenn, 2008
Cont_I	Continue	8 (SSR)	0.58	0.078	-	-	-	-	-	Bittencourt and Sebbenn, 2008
Cont_II	Continue	8 (SSR)	0.54	0.089	-	-	-	-	-	Bittencourt and Sebbenn, 2008
CJ	Continue	6 (SSR)	0.81	-0.089	25	-	-	-	-	Patreze and Tsai, 2010
CJ	Continue	7 (Alloz)	-	0.029	-	-	-	-	-	Sousa *et al*. 2005
Irati	Continue	7 (Alloz)	-	0.101*	-	-	-	-	-	Sousa *et al*. 2005
Caçador	Continue	7 (Alloz)	-	0.043*	-	-	-	-		Sousa *et al*. 2005
Três Barras	Continue	9 (Alloz)	-	0.207*	-	-	-	-	-	Ferreira *et al*. 2012
BJ	Continue	5 (SSR)	0.58	0.076*	43	0.003	-	-	-	Stefenon *et al*. 2007
FV	Continue	5 (SSR)	0.61	0.081**	0	0.009	-	-	-	Stefenon *et al*. 2007
RG	Continue	5 (SSR)	0.67	0.103***	60	0.012	-	-	-	Stefenon *et al*. 2007
CJ	Continue	5 (SSR)	0.50	0.139***	20	-0.001	-	-	-	Stefenon *et al*. 2007
Caçador	Continue	9 (SSR)	0.58	0.096*	20	0.003	-	-	-	Sant'Anna *et al*. 2013
CENI	Fragment	8 (SSR)	-	0.150*	50	-	-	-	-	Bittencourt and Sebbenn, 2007
Frag_I	Fragment	8 (SSR)	0.57	0.046	-	-	-	-	-	Bittencourt and Sebbenn, 2008

continues

TABLE 2 - RESULTS OF OBSERVED HETEROZYGOSITY (H_o), FIXATION INDEX (F), INTRAPOPULATION SPATIAL GENETIC STRUCTURE (SGS), Sp STATISTIC, POLLEN IMMIGRATION (m), DISTANCE OF POLLEN DISPERSAL (D) AND EFFECTIVE POLLINATION NEIGHBORHOOD AREA (A_{ep}) IN STUDIES OF *ARAUCARIA ANGUSTIFOLIA*. (*, ** AND *** ARE F VALUES SIGNIFICANT AT LEVELS OF 0.90, 0.95 AND 0.99, RESPECTIVELY).

continuation

Population	Kind	N locus (marker)	H_o	F	SGS (m)	Sp	m (%)	D mean (maximum) (m)	A_{ep} (ha)	Authors
Frag_III.	Fragment	8 (SSR)	0.55	0.081	-	-	-	-	-	Bittencourt and Sebbenn, 2008
Frag_IV	Fragment	8 (SSR)	0.48	0.175*	-	-	-	-	-	Bittencourt and Sebbenn, 2008
Group_I	Group_I	8 (SSR)	0.53	0.123	-	-	-	-	-	Bittencourt and Sebbenn, 2008
Group_II	Group_II	8 (SSR)	0.52	0.068	-	-	-	-	-	Bittencourt and Sebbenn, 2008
Group_III	Group_III	8 (SSR)	0.56	0.054	-	-	-	-	-	Bittencourt and Sebbenn, 2008
Group_III	Group_IV	8 (SSR)	0.48	0.072	-	-	-	-	-	Bittencourt and Sebbenn, 2008
CJ	Exploited	7 (Alloz)	-	-0.116*	-	-	-	-	-	Sousa *et al*. 2005
Canoinhas	Planted	9 (Alloz)	-	0.184*	-	-	-	-	-	Ferreira *et al*. 2012
Anita Garibaldi	Planted	9 (Alloz)	-	0.312*	-	-	-	-	-	Ferreira *et al*. 2012
Curitibanos	Planted	9 (Alloz)	-	0.273*	-	-	-	-	-	Ferreira *et al*. 2012
Variation			0.48-0.81	-0.116-0.312	25-75	-0.001-0.016				

continues

TABLE 2 - RESULTS OF OBSERVED HETEROZYGOSITY (H_o), FIXATION INDEX (F), INTRAPOPULATION SPATIAL GENETIC STRUCTURE (SGS), Sp STATISTIC, POLLEN IMMIGRATION (m), DISTANCE OF POLLEN DISPERSAL (D) AND EFFECTIVE POLLINATION NEIGHBORHOOD AREA (A_{ep}) IN STUDIES OF *ARAUCARIA ANGUSTIFOLIA*. (*, ** AND *** ARE F VALUES SIGNIFICANT AT LEVELS OF 0.90, 0.95 AND 0.99, RESPECTIVELY).

continuation

Population	Kind	N locus (marker)	H_o	F	SGS (m)	Sp	m (%)	D mean (maximum) (m)	A_{ep} (ha)	Authors
JUVENILES										
Três Barras	Continue	9 (alloz)	-	0.014	-	-	-	-	-	Ferreira *et al*. 2012
Caçador	Continue	9 (SSR)	0.53	0.163*	20	0.004	6	134 (343)	-	Sant'Anna *et al*. 2013
CENI	Fragment	8 (SSR)	-	0.124*	50	-	7	75 (1792)	1.2	Bittencourt and Sebbenn, 2007
Canoinhas	Planted	9 (Alloz)	-	0.010	-	-	-	-	-	Ferreira *et al*. 2012
Anita Garibaldi	Planted	9 (Alloz)	-	0.167*	-	-	-	-	-	Ferreira *et al*. 2012
Curitibanos	Planted	9 (Alloz)	-	0.020	-	-	-	-	-	Ferreira *et al*. 2012
CENI	Fragment	8 (SSR)	-	0.197*	50	-	3	70 (1779)	1.0	Bittencourt and Sebbenn, 2007
Variation				0.010-0.167	20-50		3-7	70-134	1.0-1.2	
SEEDS										
Manguerinha	Continue	8 (SSR)	0.55	0.052	-	-	54	102 (344)	1.7	Bittencourt and Sebbenn, 2008
Três Barras	Continue	9 (Alloz)	-	-0.029	-	-	-	-	-	Ferreira *et al*. 2012
CENI	Fragment	8 (SSR)	-	0.082*	-	-	4	83 (2006)	1.7	Bittencourt and Sebbenn, 2007
Group_I	Isolated tree	8 (SSR)	0.46	-0.031	-	-	55	-	-	Bittencourt and Sebbenn, 2009

continues

TABLE 2 - RESULTS OF OBSERVED HETEROZYGOSITY (H_o), FIXATION INDEX (F), INTRAPOPULATION SPATIAL GENETIC STRUCTURE (SGS), Sp STATISTIC, POLLEN IMMIGRATION (m), DISTANCE OF POLLEN DISPERSAL (D) AND EFFECTIVE POLLINATION NEIGHBORHOOD AREA (A_{ep}) IN STUDIES OF *ARAUCARIA ANGUSTIFOLIA*. (*, ** AND *** ARE F VALUES SIGNIFICANT AT LEVELS OF 0.90, 0.95 AND 0.99, RESPECTIVELY).

conclusion

Population	Kind	N locus (marker)	H_o	F	SGS (m)	Sp	m (%)	D mean (maximum) (m)	A_{ep} (ha)	Authors
Group_II	Isolated tree	8 (SSR)	0.57	-0.081	-	-	50	-	-	Bittencourt and Sebbenn, 2009
Group_III	Isolated tree	8 (SSR)	0.53	-0.007	-	-	84	-	-	Bittencourt and Sebbenn, 2009
CENI	Isolated tree	8 (SSR)	-	-	-	-	6	25 (1913)	0.3	Bittencourt and Sebbenn, 2007
Canoinhas	Planted	9 (Alloz)	-	0.002	-	-	-	-	-	Ferreira *et al*. 2012
Anita Garibaldi	Planted	9 (Alloz)	-	0.034	-	-	-	-	-	Ferreira *et al*. 2012
Hand-pollinated	Female	8 (SSR)	0.66	-0.258*	-	-	-	-	-	Danner *et al*. 2013
Hand-Pollinated	Monoecious	8 (SSR)	0.36	0.175*	-	-	-	-	-	Danner *et al*. 2013
Open-pollinated	Female	8 (SSR)	0.83	-0.229*	-	-	-	-	-	Danner *et al*. 2013
Open-pollinated	Monoecious	8 (SSR)	0.52	-0.049	-	-	-	-	-	Danner *et al*. 2013
Variation			0.36-0.83	-0.258-0.175			6-55	23-102	0.3-1.7	

SOURCE: The author.

CHAPTER V: FINAL CONSIDERATIONS

In relation to the procedure developed for sampling tissues from tall trees aiming at DNA isolation, the methodology described in Chapter II is faster and safer for sample collection in the field in comparison to traditional leaf sampling methods. By correctly sampling and storing vascular cambium tissue, the quality and quantity of DNA required for molecular studies can be maintained, thus significantly reducing the costs and risks involved in collecting and analyzing samples from tall trees in a wide range of forest conditions.

The forest Cluster where a census of adult *A. angustifolia* trees was carried out did not show signs of regeneration, confirming the advanced successional stage of this study area. This adult *A. angustifolia* population presented high levels of genetic diversity and an absence of biparental inbreeding. Additionally we verified the existence of SGS up to 100 m, which can explain the low effective population size of this population. Moreover, the results of the pollen flow analysis showed that the population size is greater than the studied area, confirming the existence of functional connections between the Cluster and other adjacent forest areas. These characteristics highlight the importance of forest scenarios for conservation purposes. Forest clusters retain high levels of genetic diversity, indicating the existence of genetic mechanisms that naturally deter mating among related individuals.

The diversity of seeds sampled in the four contrasting population scenarios also showed that small populations are able to maintain high levels of genetic diversity and low levels of relatedness if located close to larger populations. The relatively high allelic richness and exclusive alleles in smaller, managed and relatively isolated fragments (i.e., Private) highlight the importance of small forest areas for species conservation. Not only can these areas retain diversity and unique alleles not present in continuous forest areas, but they can also act as a connection linking several populations and isolated trees throughout the landscape.

As such, if one considers genetic diversity as the main criterion in selecting areas for conservation, our results show that in order to conserve *A. angustifolia* and the biome's overall biodiversity, it is important to combine larger populations with smaller fragments that are not located adjacent or very close to each. Such a strategy would optimize the conservation of genetic diversity of *A. angustifolia* in a

landscape scenario.

The vast majority of forest fragments found in Southern Brazil are found on small-scale rural properties. For small-scale farmers, the prohibition of forest management (of any type) has been an economic burden, leading to a limited increase in the areas with *A. angustifolia* over the last 20 years. Considering the economic and cultural relationship that exists between small-scale farmers and *A. angustifolia*, together with the importance of small fragments for the species conservation, the development of inclusive strategies that give incentives for land owners to maintain and sustainably use their forests is crucial. Thus, the inclusion of small fragments and isolated trees in the landscape in conservation strategies through payment for environmental services will require the involvement of different stakeholders, especially small-scale land owners as the majority of Araucaria Forest fragments are located on small farms.

The results of the genetic analyses conducted in this study demonstrate that conservation strategies for *A. angustifolia* should focus not only on larger populations such as those found in large protected areas, but they should also include smaller fragments in private properties, and particularly properties located up to 2 km from continuous forests. Moreover, to retain an effective population size of 150 unrelated individuals either for conservation or breeding purposes, is necessary collect seeds from 45 trees located near forest scenarios or 56 trees located in isolated patches. Preferentially, sites included for seed collection should be located at least 5 km apart to more effectively achieve species diversity on a regional landscape scale.