UNIVERSIDADE FEDERAL DE SANTA CATARINA

PROGRAMA DE PÓS-GRADUAÇÃO EM RECURSOS GENÉTICOS VEGETAIS

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DINÂMICA DA DIVERSIDADE GENÉTICA DE *Araucaria angustifolia* (Bertol.) Kuntze EM CAMPO E FLORESTA NO SUL DO BRASIL

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Não podemos ganhar a batalha de salvar as espécies e os ambientes se não formarmos uma ligação emocional entre nós e a natureza...Temos de deixar espaço para a natureza em nossos corações.

Stephen Jay Gould

Dedico à minha mãe Maria Bertoli Cristofolini

e ao meu pai Dacio Cristofolini.

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RESUMO

No início do século XX cerca de 35% da cobertura vegetal dos estados do sul do Brasil estavam representados pela Floresta Ombrófila Mista, na qual a *Araucaria angustifolia* (Bert.) O. Kuntze foi o principal componente. Atualmente, estima-se que os remanescentes desta floresta ocupem entre 1% a 4% da área original e no estado de Santa Catarina não ultrapassando 5%, com predominância de formações secundárias. Grande parte dos povoamentos naturais de *A. angustifolia* foram devastados para a exploração da madeira. Essa exploração da floresta de Araucária aconteceu de maneira predatória e não sustentável, ameaçando a sobrevivência da espécie que hoje encontra-se na categoria de criticamente ameaçada na lista de espécies ameaçadas da União pela conservação da natureza (IUCN), necessitando-se de planos eficientes e urgentes de conservação por ser uma espécie de grande importância ecológica e econômica. E para elaborar planos efetivos de conservação tem-se a necessidade de conhecer a diversidade e estrutura genética da espécie, bem como a dinâmica desta diversidade, em diferentes áreas de ocorrência, pois a espécie se comporta de maneira diferente conforme o histórico de ocupação e a densidade da mata. Sendo assim, esta tese abordou os resultados de estudos da dinâmica da diversidade genética de três populações em paisagem de campo e floresta, e tem como objetivo fundamentar estratégias de conservação e uso para *A. angustifolia*, com base em indicadores genéticos nas duas paisagens. Para tanto, o estudo está baseado na caracterização genética de todos os indivíduos antigos e ingressantes de duas parcelas permanentes em Santa Catarina e Rio Grande do Sul, uma na região de campos de São Francisco de Paula, no Centro de pesquisas e conservação da natureza Pró-mata – PUCRS, no Rio Grande do Sul e a outra em remanescente florestal, na Floresta Nacional de Três Barras, em Santa Catarina, assim como compará-las com o primeiro estudo realizado em campo natural antropizado em propriedade particular na região da Coxilha Rica, Santa Catarina. Para tanto, foram utilizados no total nove marcadores microssatélites, testado o desequíbrio de ligação entre os locos e a presença de alelos nulos. Foram estimados os índices de diversidade genética, taxa de cruzamento e fluxo gênico histórico e contemporâneo na coorte de adultos, jovens e progênies, gerando assim, informações da dinâmica da diversidade genética no espaço e no tempo. Os resultados analisados apontam importantes diferenças na dinâmica da diversidade genética na paisagem de campo e floresta, com maior eficiência na manutenção da diversidade genética no campo, apresentando maiores distâncias de dispersão de pólen tanto na área de conservação quanto no campo antropizado e índice de fixação não diferente de zero na coorte das progênies no campo antropizado. As distâncias de dispersão de sementes foram maiores na paisagem de campo em área de conservação comparativamente a todos os estudos já realizados para espécie, possivelmente devido a presença dos dispersores da espécie na região. Portanto, estratégias de conservação diferentes para cada paisagem devem ser consideradas, como o aumento de unidades de conservação em áreas de campo, investimento em conservação da espécie em propriedades particulares, implantação de árvores isoladas ou conjunto de árvores para conexão de fragmentos, reflorestamento com a espécie e coleta de sementes em áreas abertas para produção de mudas em planos regionais de conservação.

Palavras-chave: microsatélites, fluxo de pólen, fluxo de sementes, conservação.

Abstract

Beginning of the 20th century, about 35% of the vegetation cover of the Southern Brazilian states were represented by the Mixed Ombrophilous Forest, in which *Araucaria angustifolia* (Bert.) O. Kuntze is the main component. Currently, it is estimated that the remnants of this forest have among 1% and 4% of the original area and it not exceeding 5% in the state of Santa Catarina. Most of *A. angustifolia* populations were devastated for the wood exploitation. The Araucaria forest exploitation happened in a predatory and unsustainable way, threatening the survival of the species that today is critically endangered in the list of endangered species of the International Union for Conservation of Nature (IUCN), being necessary urgent and efficient plans for conservation for this species of great ecological and economic importance. It is necessary to understand the diversity and structure genetic for a effective conservation plan, as well as the dynamics of this diversity in different areas of occurrence, since the species grows differently according to the history occupation and density. Thus, the thesis approached the evaluation of the dynamics of the genetic diversity of three populations in natural grassland and forest landscape and aims to support conservation and use strategies for *A. angustifolia* based on genetic indicators. Therefore, the study is basing on the genetic characterization of all the old and incoming individuals from two permanent plots in Santa Catarina and Rio Grande do Sul, one in grassland region in São Francisco de Paula, at the Center for research and conservation of nature Pro-Mata in the state of Rio Grande do Sul and the other in forest remnants in the Três Barras National Forest in Santa Catarina, as well as comparing them with the first study conducted in an anthropized grassland in the region of Coxilha Rica, Santa Catarina. Therefore, a total of nine microsatellites markers were used and tested the linkage disequilibrium and the presence of null alleles. Genetic diversity, mating system and historical and contemporary gene flow were estimated in the cohort of adults, juveniles and progenies, thus generating information on the dynamics of genetic diversity in space and time. The results showed important differences in the dynamics of genetic diversity in the grassland and forest landscape, with greater efficiency in the maintenance of genetic diversity in grassland, presenting higher values of distances of pollen dispersal both grasslands than the forest and fixation index not different from zero in the progeny cohort in the anthropic grassland. Seed dispersal distances were higher in grassland landscape in conservation area, possibly due to the presence of the natural dispersers of the species. Therefore, it is necessary conservation strategies for each landscape, such as increasing conservation units in natural grassland areas, investing in conservation of the species in private properties, implantation of isolated trees or set of trees connection forest fragments, species reforestation and collection of seeds in open areas for seedling production in regional conservation plans.

Keywords: microsatellites, pollen flow, seed flow, conservation.

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INTRODUÇÃO E JUSTIFICATIVA

A conservação da natureza visa proteger áreas representativas de habitats e comunidades identificados e a conservação genética vai além, visando o valor da riqueza das diferenças genéticas (Mcneely et al.1990). Informações sobre diversidade genética, estrutura populacional, sistema de cruzamento, endogamia, fluxo gênico e dispersão ao longo do tempo são valiosos na avaliação da vulnerabilidade de populações e espécies (Kettle and Koh 2014). Espécies com fluxo gênico limitado podem ser particularmente vulneráveis aos efeitos negativos da fragmentação florestal e ao pequeno tamanho efetivo populacional (Finger et al. 2012).

Informações sobre as distâncias de dispersão do pólen são importantes para uma melhor compreensão dos processos populacionais das espécies de plantas, o que pode auxiliar tanto no desenvolvimento de estratégias de conservação, como em programas de coleta de sementes para reflorestamento ou restauração e uso sustentável de espécies vegetais em seus ambientes naturais (Ellstrand and Elam 1993; Ellstrand 2014; Degen and Sebbenn 2014). Em plantas, o fluxo gênico tem consequências importantes para questões aplicadas em que a presença ou ausência de fluxo de genes pode influenciar o resultado de uma decisão política, reguladora ou de manejo (Ellstrand 2014). O fluxo gênico é um dos fatores mais importantes que moldam a estrutura genética das populações e pode ser considerado benéfico ou deletério do ponto de vista da conservação genética (Burczyk et al. 2004). O isolamento genético em plantas refere-se geralmente à falta de movimento de pólen entre sítios porque para a maioria das espécies de plantas o pólen é o modo mais importante de fluxo de genes (Ellstrand 1992).

No sul do Brasil, a espécie ameaçada *Araucaria angustifolia* tem sido foco de estudos relacionados a estas questões devido a sua importância ecológica, mas também econômica. A espécie em estudo, única no país, pertence a família Araucariaceae, a qual compreende atualmente três gêneros: *Wollemia*, com uma espécie restrita ao Sudeste da Austrália, *Agathis*, com 13 espécies exclusivas da Australásia e *Araucaria*, com 19 espécies, sendo duas sulamericanas: *Araucaria angustifolia* e *Araucaria araucana* (Kershaw and Wagstaff 2001).

A espécie é componente marcante da fitofisionomia da Floresta Ombrófila Mista, pertence a uma região de clima subtropical, em altitudes que variam de 500 a 1200 metros. Sua distribuição era contínua nas porções mais elevadas do Planalto Meridional, nos Estados do Paraná, Santa Catarina e Rio Grande do Sul. Além disso, ela ocorre, descontinuamente, no Estado de São Paulo, nas serras de Paranapiacaba, Cantareira e Campos do Jordão; em Minas Gerais, nas regiões de Poços de Caldas e na Serra da Mantiqueira; no Rio de Janeiro, no maciço de Itatiaia e na Serra da Bocaina (Klein, 1960; Reitz, 1966); além do leste da Província de Misiones, Argentina (Mattos 1994).

A vegetação da Floresta Ombrófila Mista não constitui uma formação homogênea, mas é formada por diversos tipos de submatas, constituídas por árvores características nas diferentes áreas de ocorrência, ocorrendo também em campos naturais na forma de capões arbustivos ou árvores isoladas, sempre em expansão. De acordo com (Klein 1978), são cinco formações vegetacionais distintas em Santa Catarina: 1) no Planalto Norte e Meio-oeste associada principalmente a *Ocotea porosa* e *Ilex paraguariensis*, 2) no Planalto Sul a *Ocotea puchella* e 3) *Nectandra lanceolata* e no Extremo-oeste a *Apuleia leiocarpa* e *Parapiptadenia rigida*. Ainda no 4) Extremo-oeste e em regiões de transição com a Floresta Ombrófila Densa, associada a faxinais e 5) em formações de campos, formando os capões.

Os campos no Sul do Brasil, também conhecidos como campos do Planalto, são caracterizados em campos limpos, sujos ou por florestas de transição como faxinais, caivas e catanduvas (Klein, 1978). Especificamente nos campos com capões predominam os agrupamentos herbáceos formados por Gramíneas, Ciperaceas, Compostas, Leguminosas e Verbenaceas (campos limpos) assim como os capões e as matas ciliares e de galeria formando os bosques de araucária no meio da formação campestre (Klein, 1978). Os capões são formações naturais de ilhotas de matas espalhadas pelos campos, distinguindo-se pela forma e arquitetura de suas bordas bastante variáveis (Mattos, 1994).

Desde o último máximo glaciar a espécie expandiu sua área de ocorrência a partir de vales para uma grande área de campos naturais no Sul do Brasil, favorecida pelas mudanças climáticas do holoceno tardio (Behling et al. 2009) e pela ação dos grupos humanos no último milênio (Bitencourt and Krauspenhar 2006; Behling et al. 2009; Reis et al.

2014). Assim, parte de sua área de ocorrência inclui as florestas densas e parte de sua área de ocorrência inclui ainda áreas de campo, com capões/manchas com presença dominante de araucária (Mattos 1994). Porém, a exploração madeireira e expansão das fronteiras agrícolas ao longo do século XX reduziram a área com cobertura floresta da FOM a 12.6% (Ribeiro et al. 2009) da sua cobertura original, que se encontra na forma de fragmentos e, em grande maioria, cobertos com formações secundárias em fase inicial ou intermediária de sucessão.

A exploração da floresta de *A. angustifolia*, desde o início da colonização européia, alavancou o desenvolvimento para a região sul do Brasil, mas ocorreu de maneira predatória e não sustentável, seja do ponto de vista social, econômico e ecológico (Guerra et al. 2002), fazendo com que a espécie permaneça desde 1997 na lista vermelha da União Internacional para Conservação da Natureza (IUCN) (Thomas 2013), na lista Brasileira de espécies ameaçadas de extinção desde 1994 (IN / IBAMA 1994 and IN 06/MMA 2008). Além disso, possui apenas 0.62% de sua área de ocorrência em áreas protegidas (Indrusiak and Monteiro 2009) e praticamente nenhuma área de ambiente de campo com capões protegido.

Especificamente, os campos no Sul do Brasil ocupam cerca de 13,7 milhões de hectares e suportam níveis muito elevados de biodiversidade e, apesar da sua elevada riqueza de espécies, os campos não são adequadamente protegidos pelas políticas de conservação (Overbeck et al. 2007). Nas últimas três décadas, aproximadamente 25% da área de campo foi perdida devido a mudanças no uso da terra, no entanto, a representação dos campos em unidades de conservação é extremamente baixa (menos de 0,5% (Overbeck et al. 2007) e a gestão na maioria delas é inadequada para preservação.

Campo e Floresta com *A. angustifolia* formam hoje um mosaico na paisagem do Planalto no Sul do Brasil, porém os campos são mais antigos (Pillar et al. 2009b). Mesmo assim a ênfase conservacionista tem sido geralmente dirigida às comunidades florestais, sendo os ecossistemas de campos muitas vezes vistos como antrópicos, por simples desconhecimento sobre as formações vegetais naturais do sul do Brasil (Pillar et al. 2009b). A flora dos campos sulinos é extremamente rica e portanto, devido à alta diversidade de espécies e endemismos da flora e fauna, o esforço para a conservação dos campos deve ser tanto quanto aquele de formações florestais do mesmo bioma (Pillar et al. 2009b).

Somado a isto, as informações genéticas, incluindo fluxo de pólen e estimativas de taxa de cruzamento existentes para a espécie são provenientes de ambientes de floresta em diferentes fases sucessionais (Bittencourt and Sebbenn 2007; Bittencourt and Sebbenn 2008a; Sant’Anna et al. 2013; Medina-Macedo et al. 2015), inexistindo informações em ambientes de campo. Além disso, para implementar um plano de conservação efetivo e estabelecer um programa de restauração para *A. angustifolia* é necessário ter ao menos o conhecimento da distância de dispersão de pólen e da estrutura genética das populações (Reis 1996; Bittencourt and Sebbenn 2007; Sant’Anna et al. 2013). Informações relativas a níveis de diversidade genética, distribuição da variabilidade genética, taxa de cruzamento e fluxo gênico em populações naturais obtidas em diferentes populações, permite uma caracterização efetiva da dinâmica da movimentação dos alelos, permitindo projeções mais realistas de eventos (Reis 1996).

Estimativas de fluxo gênico contemporâneo tem sido obtidas para muitas espécies, como *Quercus macrocarpa* (Dow and Ashley 1996; Craft and Ashley 2010), *Eucalyptus wandoo* (Byrne et al. 2008), *Ficus arpazusa* (Nazareno and de Carvalho 2009), *Quercus robur* (Buschbom et al. 2011), *Copaifera langsdorffii* (Sebbenn et al. 2011b), incluindo coníferas como *Araucaria angustifolia* (Bittencourt and Sebbenn 2007; Bittencourt and Sebbenn 2008a; Sant’Anna et al. 2013; Medina-Macedo et al. 2015), *Pinus densiflora* (Iwaizumi et al. 2013), e *Pinus sylvestris* (Robledo-Arnuncio and Gil 2005).

Especificamente para a *A. Angustifolia*, estudos vêm sendo realizados nas últimas décadas na busca de informações para a conservação da espécie no Brasil, demonstrando diferentes resultados (tabela 1): população em equilíbrio de Hardy-Weinberg (Shimizu 2001); alta estruturação genética em uma população (Mantovani et al. 2006b); baixos níveis de diversidade genética em algumas populações (Ferreira et al. 2012); altos índices de fixação em 31 populações avaliadas (Reis et al. 2012); maior variabilidade genética contida dentro das populações (Auler et al. 2002); populações de adultos com baixa divergência genética atribuida ao transporte antropogênico de sementes e transporte de pólen em um modelo de trampolim entre os fragmentos (Stefenon et al. 2007); níveis mais elevados de estrutura genética espacial na população com maior quantidade de indivíduos juvenis (Stefenon et al. 2009); estrutura genética espacial significativa em distâncias até 75 m e alta proporção de acasalamentos biparentais (Bittencourt and Sebbenn 2008b); maior diversidade genética em populações remanescentes de florestas contínuas do que nas populações fragmentadas ou isoladas naturalmente (Souza et al. 2009); estrutura significativa até 25 metros (Patreze and Tsai 2010); fluxo de pólen mais efetivo em áreas aberta e em árvores próximas as bordas em floresta (Medina-Macedo et al. 2015); aumento de endogamia em progenies, indicando fluxo gênico ineficiente (Bittencourt and Sebbenn 2008a; Costa et al. 2015; Medina-Macedo et al. 2015) e por fim troca eficiente de alelos entre capões, tanto em relação ao fluxo de pólen como de sementes são maiores no campo do que em ambiente florestal, mantendo a diversidade genética da espécie e reduzindo a endogamia a zero (Cristofolini 2013).

Neste contexto, informações consistentes sobre a dinâmica da diversidade genética são necessárias para a estruturação de planos efetivos de conservação e uso para *A. angustifolia* visando a compreensão de como ocorrem e variam os processos de fluxo de pólen e sementes, a partir de informações de paternidade, taxa de cruzamento e diversidade em diferentes ambientes, podendo auxiliar na manutenção da dinâmica da espécie em ambientes com história de ocupação e vida evolutivamente diferentes.

Nesse sentido, esta tese teve o objetivo de avaliar a dinâmica da diversidade genética em populações de *A. angustifolia* em paisagem de campo e de floresta no Sul do Brasil. O trabalho aborda variações no sistema reprodutivo, diversidade genética e nos elementos de fluxo gênico e visa obter avanços na discussão de estratégias de conservação e uso da espécie em paisagens distintas. Assim, a tese está estruturada com esta introdução, capítulo 1 e 2 com dois artigos e o capítulo 3 integrando todos os resultados obtidos e uma síntese dos avanços alcançados, bem como perspectivas e aplicações dos resultados.

O primeiro capítulo compara a dinâmica de duas paisagens naturais da espécie, campo antropizado e floresta em unidade de conservação, com o objetivo de compreender as diferenças da espécie nas duas paisagens para desenvolvimento de estratégias de conservação. Para tanto, foi realizada a análise da diversidade genética, fluxo gênico e sistema de cruzamento de duas populações a partir de marcadores microssatélites. A população de campo encontra-se na região da Coxilha Rica, em Lages, no estado de Santa Catarina e a população em floresta na cidade de Três Barras, em Santa Catarina.

O segundo capítulo trata da avaliação da dinâmica da diversidade genética em paisagem de campo dentro de unidade de conservação abordando as seguintes questões: 1. A paisagem de campo dentro de unidade de conservação reduz a diversidade genética nas gerações mais recentes? 2. O fluxo gênico é capaz de manter a diversidade genética no campo? 3. Existe contribuição de alelos migrantes da floresta contínua circundando o campo? Para isso, realizou-se a análise da diversidade genética, fluxo de pólen e de sementes e o sistema de cruzamento através de marcadores microssatélites. A população amostrada faz parte de uma área inteira de campo natural no Centro de pesquisas e conservação da natureza Pró-mata, em São Francisco de Paula no estado do Rio Grande do Sul.

Tabela 1. Lista de estudos genéticos realizados com diferentes marcadores nos últimos anos para a espécie *Araucaria angustifolia* em áreas de ocorrência nos estados do Rio Grande do Sul, Santa Catarina, Paraná, São Paulo, Rio de Janeiro e Minas Gerais.

Referência	**Marcador**	**Populações**	**Locos**	**Indivíduos**	**Ho**	**He**	***f***	**A**
Schlogl 2000	RFLP	8 (SC,PR)	9	141	--	0.61	--	--
Medri et al. 2003	RAPD	3 (SC)	211	24	0.26	--	--	--
Stefenon et al. 2004	RAPD	2 (SP,SC)	40	80	0.50	--	--	--
Stefenon et al. 2003	AFLP	1 (SC)	62	30	0.54	--	--	--
Sousa 2006	AFLP	5 (RJ,RS,PR,MG)	683	243	0.2	--	--	--
Stefenon et al. 2007	AFLP	5 (SC,RS,PR)	166	320	0.21	--	--	--
Shimizu et al. 2001	Isoenzimas	1(PR)	8	120	0.24	0.25	0.06	--
Auler et al. 2002	Isoenzimas	9 (SC)	15	328	0.06	0.11	0.14	33
Sousa et al. 2004	Isoenzimas	3 (SC)	24	257	0.12	0.13	--	--
Mantovani et al. 2006	Isoenzimas	1 (SP)	16	334	0.17	0.17	0.01	26
Ferreira et al. 2011	Isoenzimas	1 (SC)	9	278	0.10	0.11	0.06	26
Reis et al. 2012	Isoenzimas	31 (SC)	13	1550	0.09	0.12	0.24	37
Zechini 2012	Isoenzimas	6 (SC)	13	1540	0.076	0.079	0.03	27
Salgueiro et al. 2005	SSR	3 (RJ,PR,RS)	8	60	0.31	0.34	--	49
Stefenon et al. 2007	SSR	6 (SP,PR,SC,RS)	5	384	0.58	0.71	0.10	73
Bittencourt & Sebbenn 2007	SSR	1 (PR)	8	228	0.51	0.59	0.14	77
Bittencourt & Sebbenn 2008	SSR	1 (PR)	8	298	0.55	0.59	0.06	64

Sujii et al. 2008	SSR	2 (SC,RS)	9	262	0.68	0.83	0.19	--
Giacomini et al. 2008	SSR	1 (RS)	6	--	0.85	0.24	--	64
Bittencourt & Sebbenn 2009	SSR	1 (PR)	8	1,179	0.54	0.58	0.09	106
Patreze & Tsai 2010	SSR	1 (SP)	6	105	0.80	0.74	-0.09	70
Medina-Macedo et al. 2011	SSR	1	8	--	0.57	0.62	0.08	--
Sant'anna et al. 2013	SSR	1 (SC)	9	513	0.55	0.63	0.13	86
Cristofolini 2013	SSR	2 (SC)	8	486	0.53	0.60	0.10	87
Medina-Macedo et al. 2015	SSR	1 (SC)	8	294	0.91	0.70	0.31	--
Costa et al. 2015	SSR	1 (SC)	8	487	0.67	0.66	0.05	--
Medina-Macedo et al. 2016	SSR	1 (SC)	8	1,113	0.83	0.74	--	--

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Capítulo 1

Contemporary gene flow and mating system analyses for *Araucaria angustifolia* in two different landscapes

Abstract

In this study, we analyzed genetic diversity, gene flow by pollen, and outcrossing rates using seven microsatellite loci for two *Araucaria angustifolia* populations from two contrasting landscape environments, forest and anthropized grassland (‘campo’), in the Atlantic Forest, Southern Brazil. From the two populations, a total of 423 reproductive trees of the dioecious and anemophilous species were mapped, sexed, and genotyped. Seeds were collected from 11 seed trees in the anthropized grassland and nine seed trees in the forest, for a total of 338 progeny arrays. As expected for a dioecious plant species, mating system analyses showed that *A. angustifolia* is allogamous with t_m varying from 0.99 to 1.0. In addition, both biparental inbreeding (0.033 – 0.034) and selfing rates (0 – 0.004) were not significantly different from zero. The median pollination distance was relatively short, varying from 91 (forest population) to 126 m (grassland population). Overall, the grassland population presented pollen flow at greater distances than the forest population and the fixation index was not different from zero in progenies. These results reinforce the importance of conserving this neglected landscape due to its ability to connect forest fragments and reduce the fixation index, thus ensuring the maintenance of this endangered species, particularly in private areas.

Keywords: *Araucaria angustifolia*, conservation genetics, nuclear microsatellites, natural grassland.

INTRODUCTION

Information on pollen dispersal distances are important for developing a better understanding of plant species population processes, which can inform the development of conservation strategies, seed collection programs for reforestation or restoration, and sustainable use of plant species in their natural environments (Ellstrand and Elam 1993; Ellstrand 2014; Degen and Sebbenn 2014). Patterns of genetic transmission within and among plant populations are controlled through mating systems that use cross- and/or self-fertilization. Outcrossing promotes gene flow through pollen and reduces the likelihood of microgeographic differentiation and population substructuring (Nazareno and Reis 2012).

Estimates of contemporary gene flow by pollen has been carried out for a plethora of species, such as *Quercus macrocarpa* (Dow and Ashley 1996; Craft and Ashley 2010), *Eucalyptus wandoo* (Byrne et al. 2008), *Ficus arpazusa* (Nazareno and de Carvalho 2009), *Quercus robur* (Buschbom et al. 2011), *Copaifera langsdorffii* (Sebbenn et al. 2011b), as well as conifers such as *Araucaria angustifolia* (Bittencourt and

Sebbenn 2007; Bittencourt and Sebbenn 2008a; Sant'Anna et al. 2013; Medina-Macedo et al. 2015), *Pinus densiflora* (Iwaizumi et al. 2013), and *Pinus sylvestris* (Robledo-Arnuncio and Gil 2005). In general, these studies have emphasized forest landscapes and have not considered other landscapes where these species exist. In Brazil, plant species, such as the threatened *A. angustifolia*, can occur in different types of landscapes, including both continuous forest and *Campos* grassland. *Campos* grassland with patches ('Capoes') in southern Brazil, also known as *Planalto*, are dominate by herbaceous clusters of Gramineae, Cyperaceae, Compost, Leguminosae and Verbenaceae as well as riparian forest and gallery forests modeling the pine forests in the midst of *Campos* grassland formation (Klein, 1978) . Patches ('capoes') are natural formations of forest islands inside de *Campos* grasslands, distinguished by the variable shape and architecture of the edges (Mattos, 1994).Genetic information including gene flow by pollen and estimates of outcrossing rates for this species are all from forest in different successional stages and open area by degradation (Bittencourt and Sebbenn 2007; Sant'Anna et al. 2013; Medina-Macedo et al. 2015).

The species is a dioecious conifer from South America and it has anemophilous dispersion. Until the early twentieth century, the species occurred across a vast range of approximately 200,000 km^2 in the South and Southeast of Brazil and northeastern Argentina (Reitz and Klein 1966; Hueck 1972; Mattos 1994). It is a key species of the Mixed Ombrophilous Forest, or Araucaria Forest, which is part of the Atlantic Forest biome. Since the Last Glacial Maximum (LGM), the range of the species expanded from valleys to a large areas of natural grasslands in Southern Brazil, favored by warmer climates in the late Holocene (Behling et al. 2009). Furthermore, over the last millennium, range expansion of the species occurred as a result of human groups (Bitencourt and Krauspenhar 2006; Pillar et al. 2009a) for which the tree is a strong cultural symbol, who have played a key role in its rapid territorial expansion (Reis et al. 2014). As a result, the range of the species now includes dense forests and natural grasslands with patches dominated by *A. angustifolia* (Mattos 1994). However, the species has become critically endangered (Thomas 2013) due to uncontrolled and unsustainable exploitation of the Araucaria Forest in Southern Brazil (Guerra et al. 2002). In addition, logging and the expansion of agricultural land use throughout the twentieth century reduced the forest cover to 12.6% of its original area (Ribeiro et al. 2009). The remaining Araucaria Forests are in fragments, with most large fragments as secondary forests at initial or intermediate stages of succession (Vibrans et al. 2012). Moreover, only 0.62% of the natural range of the forest is currently within protected areas and virtually no natural *Campos* grasslands are protected by law.

Previous studies for *A. angustifolia* in degraded forest indicate that gene flow by pollen is more effective in open areas and among trees near the edges of forests which can isolate alleles within forest fragments. Thus, different conservation and restoration strategies for each environment are necessary (Medina-Macedo et al. 2015). Furthermore, studies in fragmented forest landscapes have shown increases in inbreeding in progenies (Bittencourt and Sebbenn 2008a; Costa et al. 2015; Medina-Macedo et al. 2015), which could indicate inefficient gene flow. Studies have shown the negative effects of forest fragmentation on the genetic diversity of *A. angustifolia* populations, including low levels of observed and expected heterozygosity (Auler et al. 2002), loss of rare alleles, increases in inbreeding coefficients, and high fixation index (Bittencourt and Sebbenn 2009).

Grassland landscapes could be used to support conservation strategies and avoid the extinction of the endangered *A. angustifolia*, as they are naturally open vegetation areas that occur between forest fragments, which can act as efficient gene flow corridors. In this study, we assess gene flow by pollen and outcrossing rates in contrasting landscapes within the natural range of *A. angustifolia*, expecting higher genetic diversity and gene flow through pollen in the grassland landscape.

Material and Methods

Study sites

The study was conducted in two regions in Santa Catarina State, Southern Brazil: a) Coxilha Rica (CR), in the city of Lages (28º 02' S e 50º 17' W); and b) Três Barras National Forest (TBNF) in the city of Três Barras (64 º 1' N e 13 º 34' W) (Figure 1). The Coxilha Rica study area is located in the Santa Catarina Highland Plateau (*Planalto Serrano Catarinense*) and is composed of anthropized grassland ('campo'). Altitudes range between 500 and 2000 m, with an average annual temperature below 18°C, frosts, and freezing temperatures in winter. The soil is stony, with low fertility, and the relief is broadly undulating. The study area is on private property, with grazing, mowed grassland, and a history of selective logging over the past 35 years. In this property, we installed a plot of 9.0 ha (300 x 300 m) which included different size patches and areas of riparian forest (Figure 2). Located on the North Plateau of Santa Catarina, the Três Barras National Forest is a conservation area that was established in 1944 with an area of 4458.50 ha, and a previous history of intense logging. The altitude in the study area ranges from 700 to 800m, with an average annual temperature of 17°C, and a generally flat landscape. In the TBNF, we established a plot of 2.72 ha (170 x 160 m) to sample part of the forest fragment (Figure 3). The forest population is densely closed contrasting with the open grassland population with reduced number of pines, being ideal to study the differences of Araucaria gene flow via pollen in closed and open landscape.

Needles were collected from all *A. angustifolia* adults within the established plots, for a total sample of 150 reproductive trees (93 males and 57 females) for the grassland (CR) and 273 reproductive trees (163 males and 110 females) (Figure 2) for the forest landscape (TBNF). For analysis of the progenies, we collected 152 seeds from 11 seed trees sampled in CR, and 186 seeds from 9 seed trees in the TBNF, both during reproductive events in 2011.

Fig. 1 Geographical distribution of Araucaria Forest (light gray) and the occurrence of areas of the grassland landscape (dark gray) (IBGE 2004). Study sites (Três Barras and Lages) in the state of Santa Catarina in southern Brazil are indicated by point.

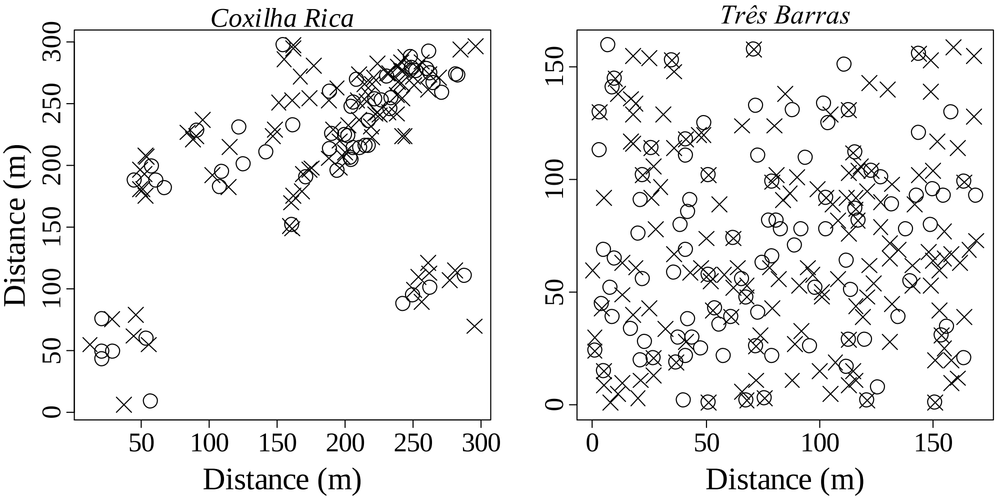

Fig. 2 Distribution of reproductive trees of *Araucaria angustifolia* (x = males, ○ = females) in permanent plots in the grassland in Coxilha Rica (300 x 300 m) and in the continuous forest in Três Barras (170 x 160 m), both populations located in Atlantic Rainforest, South of Brazil

Extraction and amplification of DNA

DNA was extracted from needles of adult trees based on the method described by Doyle & Doyle (1990). For progenies, DNA was extracted from the embryo of the seeds with the NucleoSpin Kit Plant II® (Macherey-Nagel). For DNA amplification, we used seven microsatellite loci (Table 2) and the universal M13 primer labeled with fluorochrome FAM and HEX. The reaction had a total volume of 17 ul containing 1x Taq buffer (Fermentas), 0.3 DNA polymerase U (Fermentas), 0.5 mM of forward primer, 0.2 mM of reverse primer, 0.2 uM of primer M13, 0.1 mM dNTP (Fermentas), 2.5 mM magnesium chloride (Fermentas), and 10 ng DNA. Cycles were 95 °C for 3 minutes followed by 35 cycles consisting of three steps: the first at 95 °C for 30 seconds; the second at T (a) °C for 45 seconds; and the third at 72 °C for 1 minute, with a final extension at 72 °C for 10 minutes. The amplicons were analyzed using the MegaBACE 1000 DNA Analysis System, Amersham Biosciences Corp. (GE Healthcare), diluted 3:1 with water, Tween 20 (7.75ul) and Etrox (0.25ul) (GE Healthcare). Data were analyzed based on the interpretation of electropherograms using the MegaBACE Fragment Profiler 1.2 software.

Table 2. Characteristics of microsatellite markers used for grassland and forest populations of *Araucaria angustifolia*. Ta, annealing temperature (° C); R, expected size range in base pairs (bp).

Primers	Repeat motif	T_a	R (bp)	Reference
Ag20	$(GA)_{12}$	58	257-283	Salgueiro et al. 2005
Ag23	$(TA)_5(GT)_4$	55	249-287	Salgueiro et al. 2005
Ag45	$(GT)_4AT(GT)_7$	55	170-186	Salgueiro et al. 2005
Ag56	$(TC)_{11}$	60	132-180	Salgueiro et al. 2005
Ag94	$(TC)_{12}$	55	159-203	Salgueiro et al. 2005
CRCA1	$(GA)_{19}$	55	212-232	Scott et al. 2003
CRCA2	$(GA)_{23}$	52	199-219	Scott et al. 2003

Prior Genetic analysis

Before including the microsatellite set in our study, we tested for gametic disequilibrium and presence of null alleles. As gametic disequilibrium creates pseudo-replication for analyses in which loci are assumed to be independent samples of the genome, we used the FSTAT 2.9.3.2 software (Goudet 2002) to test all loci for linkage disequilibrium, applying the Bonferroni correction for multiple comparisons (Rice 1989). Null allele frequencies were assessed for the populations using the Microchecker 2.2.3 software (Van Oosterhout et al. 2004). If significant homozygosity was detected at a given locus, it was excluded and a modified average inbreeding coefficient (F_{IS}) was calculated across all loci; its significance was estimated using a jackknife across all loci.

Genetic diversity analysis

Genetic diversity indices were averaged across all loci for all sampled adults and offspring. The estimated parameters were: average number of alleles per locus, A; allelic richness, *AR*; observed heterozygosity, H_o; and expected heterozygosity, H_e (Nei 1978). To infer the levels of inbreeding, we estimated the inbreeding coefficient (F_{IS}) and Nason's estimator of coancestry coefficient (F_{ij}), described in Loiselle et al. (1995), for each population. Significance (determined by 10,000 permutations across loci) was tested using the SPAGeDi program (Hardy and Vekemans 2002). All analyses and estimates were carried out using the FSTAT program (Goudet 2002).

Paternity analysis and gene flow by pollen

Paternity analysis was assigned by comparing the genotypes of adult males and seeds using the program CERVUS 3.0 (Kalinowski et al. 2007). The relationship was determined by Δ statistic, which is the difference between the LOD (logarithm likelihood ratio) of the most likely candidate parent minus the LOD of the second most likely candidate parent. To find the critical value of Δ (the difference in LOD scores between the two most likely parents), indicating the confidence level of paternity analysis, simulations were conducted, using 10,000 repetitions, 0.01 as the proportion of misclassified loci, and all individuals as probable father candidates for each mother tree. We used 95% as the strict confidence level and 80% as the relaxed, as suggested by Marshall et al. (1998). From the paternity analysis, pollen dispersal distance was calculated based on the position of the seed tree and the putative pollen parent within the study plots.

Determination of the mating system

The mating system was analyzed under the mixed-mating and correlated mating models, using the Multilocus mating system software MLTR, version 3.2 (Ritland 2002). The parameters estimated were: single-locus outcrossing rate ($\hat{t}_s$); multilocus outcrossing rate ($\hat{t}_m$); selfing correlation ($\hat{r}_s$); biparental inbreeding rate ($\hat{t}_m - \hat{t}_s$); multilocus paternity correlation ($\hat{r}_{p(m)}$) or proportion of full sibs among outcrossed progeny and the inbreeding coefficient of maternal parents ($\hat{F}_m$). To determine whether the values were significantly lower than 1 ($\hat{t}_m - \hat{t}_s$) or greater than 0 ($\hat{F}_m$, $\hat{t}_m - \hat{t}_s$, and $\hat{r}_{p(m)}$), a 95% confidence interval was calculated.

Other demographic and genetic parameters were assessed from the mating system parameters. The neighborhood size $\hat{N}_{ep}$, or the number of pollen donors contributing to each family, was estimated as $1/\hat{r}_{p(m)}$ (Ritland 1989). The average proportion of self-sibs ($\hat{P}_{ss}$), half-sibs ($\hat{P}_{hs}$), full-sibs ($\hat{P}_{fs}$), and self-half-sibs ($\hat{P}_{shs}$) within families was estimated as $\hat{P}_{ss} = \hat{s}^2$; $\hat{P}_{hs} = \hat{t}_{m2}(1 - \hat{r}_{p(m)})$; $\hat{P}_{fs} = \hat{t}_{m2}\hat{r}_{p(m)}$ and $\hat{P}_{shs} = 2\hat{s}\hat{t}_m$, where $\hat{s}$ (= $1 - \hat{t}_m$) is the selfing rate. The confidence intervals for these parameters were calculated based on upper and lower confidence limits estimated from mating system parameters using the MLTR program.

In addition, we calculated the coancestry coefficient among plants within progenies ($\hat{\theta}_{xy}$) from the correlation coefficient of relatedness among plants within progenies ($\hat{r}_{xy}$), as proposed by Ritland (1989): $\hat{r}_{xy} = 0.25 (1 + \hat{F}_m) [4\hat{s} + (\hat{t}_{m2} + \hat{t}_{ms} \hat{r} \hat{s}) (1 + \hat{r}_{p(m)})$. Based on the coancestry coefficient, we estimated the variance effective size as $\hat{N}_{ep} = 0.5/\hat{\theta}_{xy}$ (Cockerham 1969), considering that for diploid species the coefficient $\hat{\theta}_{xy}$ is half the coefficient $\hat{r}_{xy}$ (Lynch and Walsh 1998). In addition, Nason's kinship coefficient estimator (f_{ij} or coefficient of coancestry) described in Loiselle et al. (1995) was performed for maternal trees using the software SPAGeDi (Hardy and Vekemans 2002). The coefficient of inbreeding in embryos ($\hat{F}_o$) was inferred by calculating the fixation index using the FSTAT program (Goudet 2002). To test if $\hat{F}_o$ was significantly different from 0, we performed 10,000 permutations of alleles among individuals. The total coefficient of inbreeding $\hat{F}_o$ (= $\hat{F}_s + \hat{F}_{tm-\mathrm{ts}}$) in embryos was split into those resulting from self-fertilization ($\hat{F}_s = 0.5\hat{s} (1 + \hat{F}_m)$; Barrett et al. 1991) and mating among relatives ($\hat{F}_{tm-ts}$).

Results

Genetic analysis

For the genotypic disequilibrium analysis, none of the loci pair combinations showed significant deviation ($p < 0.001$) after Bonferroni correction. Thus, the loci could be used for all genetic analyses. In the grassland population, we found significant frequencies of null alleles for the loci AG23 and AG45 for adults and no loci with null alleles in progenies. On the other hand, for adults in the forest area, we found null alleles for loci AG94, AG20, AG23, CRCA1, CRCA2, and AG45, and among progenies in loci AG20, AG23, AG45, and CRCA1.

We found a total of 80 and 89 alleles for the grassland and forest population, respectively. The expected heterozygosity for grassland adults and progenies was 0.59 and 0.60, respectively, and the observed heterozygosity was 0.53 and 0.58 (Table 3). For the forest population, the expected heterozygosity was 0.64 for adults and 0.60 for progenies, and the observed heterozygosity was 0.55 and 0.54. Except for progenies from the grassland, the values of F_{IS} were significant and different from zero for all populations (Table 3). The allelic richness was greater in the forest population than in the grassland and in progenies compared to adults.

Table 3. Genetic diversity indices and inbreeding coefficient in the population of forest and grassland for two generations (adults and progenies) of *Araucaria angustifolia*. n is the sample size, AR the allelic richness, He the average expected heterozygosity in Hardy-Weinberg equilibrium (CI: confidence interval calculated by 1000 bootstraps), Ho the average observed heterozygosity (CI: confidence interval calculated by 1000 bootstraps), and F_{IS} is the fixation index (* p< 0,05).

	Grassland					Forest				
	n	AR	He	Ho	F_{is}	N	AR	He	Ho	F_{is}
Adults	140	9.48	0.61(0.59-0.63)	0.56(0.54-0.60)	0.1*	267	8.64	0.63(0.62-0.64)	0.56 (0.54-0.58)	0.13*
Progenies	147	8.35	0.63(0.61-0.64)	0.62(0.58-0.63)	0.03	155	9.41	0.60(0.58-0.62)	0.53(0.51-0.56)	0.10*
Average		8.91	0.62	0.59	0.03		9.02	0.62	0.55	0.11

Paternity assignment and gene flow by pollen

In the grassland population, the pollen dispersal distance obtained through paternity analysis ranged from 10 to 334 m with a median of 137 m and an average of 126 m (95% CI: 111.5 to 139.8 m). For the forest population, the dispersal distance varied from 9 to 206 m with a median of 91 m and an average of 91 m (95% CI: 84-98 m) (Figure 3).

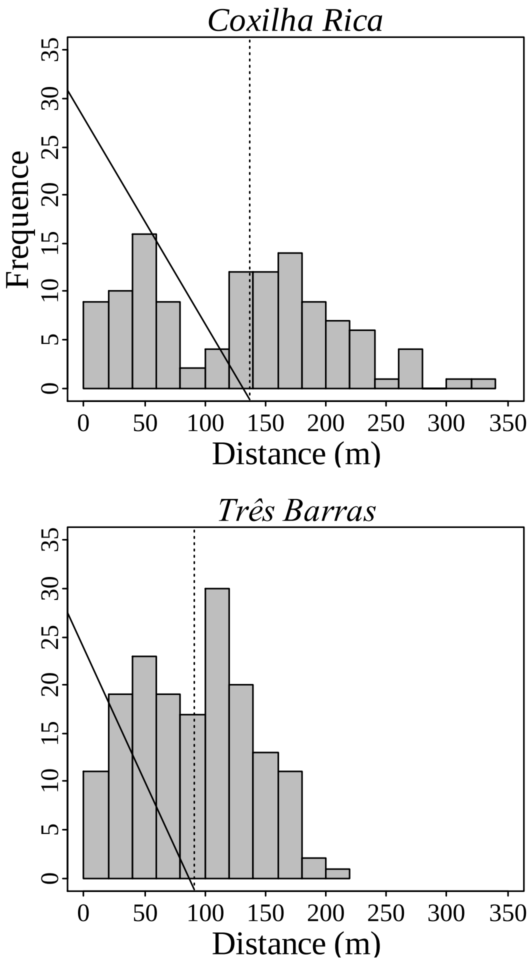

Fig. 3 Frequency of pollen dispersal distances in the grassland (*Coxilha Rica*) and Forest (*Três Barras*) populations of *Araucaria angustifolia*. Dotted line is the data median (136.7 m in grassland and 91.1 m in forest).

Mating system

For the grassland, the multilocus outcrossing rate ($\hat{t}_m$) was 1.0 and single locus outcrossing rate ($\hat{ts}$) was 0.97; for the forest, the rates were $\hat{t}_m$ = 0.99 and $\hat{t}_s$ = 0.96. The difference between these rates is low, but significantly different from zero for grassland ($\hat{t}_m - \hat{t}_s = 0.03$) and forest landscapes ($\hat{t}_m - \hat{t}_s = 0.03$), suggesting a limited rate of mating among relatives. The estimate of the correlation of selfing ($\hat{r}_s$) was low and significantly different from 0 ($\hat{r}_s = 0.104$, $P < 0.05$), indicating limited variation in the individual self-fertilization rate in this population. The $\hat{r}_{p(m)}$ value indicates that more than 16 fathers ($\hat{N}_{ep} = 16.39$) contributed to individual progeny arrays in the grassland population and more than 23 fathers ($\hat{N}_{ep} = 23.80$) in the forest population. The fixation index of mother plants ($\hat{F}_m$) estimated for the progenies was nil, indicating an absence of inbreeding in the sampled mother trees. The mean coefficient of coancestry within progenies ($\hat{\theta}_{xy}$) was 0.132 in the grassland and 0.107 in the forest. These values were lower than expected for full-sib families (0.25) (Table 4).

Table 4. Mating system parameters, estimates of inbreeding and relatedness of *Araucaria angustifolia* grassland and forest population in the Atlantic Rainforest, Santa Catarina State, Southern Brazil.

Mating system		
Population	Grassland	Forest
Multilocus outcrossing rate	1	0.996
Single-locus outcrossing rate	0.966	0.963
Selfing rate	0	0.004
Mating among relatives	0.034	0.033
Correlation of selfing	0.11	-0.999
Multilocus paternity correlation	0.061	0.042
Effective number of pollen donors	16.39	23.8
Inbreeding and genetic structure		
Inbreeding coefficient of maternal parents	0	-0.177
Inbreeding coefficient of progeny	0.03	0.107
Inbreeding in progeny from selfing	0	0.001
Inbreeding in progeny from mating among relatives	0	0.0001
Proportion (%) of self-sibs pairs	0	0.000016
Proportion (%) of half-sibs pairs	0.939	0.95
Proportion (%) of full-sibs pairs	0.061	0.041
Proportion (%) of self-half-sibs pairs	0	0.007
Coancestry within offspring	0.132	0.107
Effective size of variance	3.77	4.672

Discussion

Unlike the forest landscape, the grassland has a naturally lower density of adult individuals because they are established in patches surrounded by grassland vegetation. In this study, the densities differ markedly in each landscape, with a high density of adults in the forest (100 ind.ha^{-1}), and a low density in the grassland (16.6 ind.ha^{-1}). Furthermore, the forest presented a much higher density than is commonly reported for the species, such as 40.3 ind.ha^{-1} in a remnant of continuous forest in Santa Catarina, Brazil (Sant'Anna et al. 2013), 45 ind.ha^{-1} in the Santa Catarina Forest Inventory (Meyer et al. 2013), and 32 ind.ha^{-1} and 55 ind.ha^{-1} in a forest reserve in São Paulo, Brazil (Solórzano-Filho 2001; Mantovani et al. 2006a). Likewise, the contrast in the density of reproductive females for the grassland (6.3 ind.ha^{-1}) and for the forest (40 ind.ha^{-1}) is evident. The latter is higher in comparison to the 18 ind.ha^{-1} and 13.9 ind.ha^{-1} reported for forest reserves in São Paulo and Santa Catarina (Mantovani et al. 2006a; Paludo et al. 2009a). The ratio between female to male *A. angustifolia* individuals was 1:1.6 (57/93) for the grassland and 1:1.4 (110/162) for the forest. These values are consistent with those reported for the species in continuous forest and forest fragments (1:1.1 Paludo et al. 2009; 1:1.2, Mantovani et al. 2006; and 1:1.1, Mantovani et al. 2004), demonstrating the characteristic of a higher percentage of males than females (Mattos 1994). Forests that are particularly dense, such as the Araucaria Forest studied herein, may restrict pollen dispersal due to the existence of physical barriers that limit the movement of wind (Medina-Macedo et al. 2015); the wind can reach the top and edges of the forests, but the interior is protected (Mattos 1994).

The heterozygosity values found herein show that the grassland has a trend in maintaining genetic diversity, with Ho = 0.58 in progenies and Ho = 0.53 adults. On the other hand, progenies from the forest presented Ho = 0.54 and adults Ho = 0.55. Likewise, in the grassland population we found a reduction in fixation index for the progeny generation, which was not significant, suggesting limited crossing between related individuals. This result differs from our observations for the forest population in this study, as well as in other studies (Bittencourt and Sebbenn 2008a; Medina-Macedo et al. 2015). Our results show the potential of allelic exchange and recombination in progenies present in the grassland landscape, which together with the significant number of regenerants in the population, may provide improved capacity for species adaptation and expansion into new environments. Similarly, a study of an extremely endangered species (*Dianthus seguieri* ssp. Glaber) in a grassland in Bavaria, Germany, showed the population had high genetic variability, seed viability, and germination capacity, thus offering promising characteristics for conservation (Busch and Reisch 2016). Herein, the grassland population shows allelic exchange at greater distances than in the forest, with a greater proportion of different pollen donors (17.6%) than in the forest (14.7%), in relation to the number of males in the grassland (n = 93) and in the forest (n = 162). This difference between the two populations is likely the result of the naturally open environment in the grassland that is more exposed to winds. In addition, the number of pollen donors found in this study differs from other studies on the species: 6.4 (Bittencourt and Sebbenn 2008a) and 12.6 (Bittencourt and Sebbenn 2007). The combination of individual longevity, high intra-population genetic diversity, and the potential for high rates of pollen flow should make tree species especially resistant to extinction and the loss of genetic diversity due to changing environmental conditions (Hamrick

2004), thus making the grassland landscape an alternative environment to support the conservation of the endangered *A. angustifolia*.

Studies have not only confirmed the expansion of this species into grassland, but have also demonstrated the importance of *A. angustifolia* as a nucleation species in the grassland matrix of the high altitude plateaus in Brazil (Dos Santos et al. 2011). The expansion of forests over natural grasslands is observed in many parts of the world (Korndörfer et al. 2015) and forest patches in natural grassland landscapes are always expanding (Behling et al. 2009). However, it is important to emphasize that this naturally open environment should not be compared to forest fragmentation, where genetic drift in subsequent generations may occur (Hamrick 2004), although the environment is also exposed to winds. Furthermore, fragmentation has several serious genetic consequences which can undermine the viability of species in fragmented landscapes (Kettle and Koh 2014). Evidence of the effects of fragmentation on *A. angustifolia* include the loss of allelic frequency and rare alleles in fragments and isolated trees (Bittencourt and Sebbenn 2009), and for *Araucaria nemerosa*, in a highly fragmented environment, studies have shown a loss of rare alleles and high levels of endogamy (Kettle et al. 2007a).

The results of effects of fragmentation are different what we observed in the grassland landscape in this study, suggesting the difference between forest fragments and natural grassland, as well as the importance of connecting fragments of continuous forests through gene flow corridors. Our results highlight the need to protect these neglected grassland landscapes particularly in private proprieties that have been affected by invasion of species from nearby eucalyptus and pine plantations (Cordero et al. 2016), planted pastures (Grau et al. 2015), agriculture and cultivation of exotic species (Henderson et al. 2016), resulting in the loss of 84 to 88% of the original landscape cover (Ribeiro et al. 2009) and low richness of native species (Cordero et al. 2016). Plant population size and land use abandonment should be considered to ensure the long term protection of endangered plant species because these processes can have a significant impact on genetic variation and species' performance (Busch and Reisch 2016), as mowing events cause significant declines in the genetic diversity and/or reproductive success in species (Nakahama et al. 2016). For example, ten out of the 13 endemic Araucaria species in New Caledonian (77%) are threatened with extinction in the wild because of open cast mining, fires, and habitat destruction (Jaffré et al. 2010).

In a previous study, Costa et al. (2015) reported that patches of *A. angustifolia* interspersed among *Pinus* plantations showed fixation indexes from inbreeding which differ from areas not surrounded by plantations, indicating barriers to pollen flow and decreased migration of alleles between patches. These results reinforce the importance of developing conservation strategies and alternate land use strategies in these environments, such as reforestation with *A. angustifolia*, and partial exploitation to generate profits in particular areas. Furthermore, the substitution of exotic tree plantations with native *A. angustifolia* plantations, if properly managed, could gradually reduce the number of invasive species propagules in grassland landscapes (Barbosa et al. 2009; Cordero et al. 2016). *A. angustifolia* plantations have a high potential as colonization sites by native woody species, even their structural differences in relation to native forests (Mendonça-Lima et al. 2014) and the species act as an effective nucleation species that nurses seedling development (Korndörfer et al. 2015) becoming a positive way for the recovery of degraded areas.

Considering the current situation in Southern Brazil, where only 9.3% of Atlantic Forest (Ribeiro et al. 2009) and only 0.62% of Araucaria Forests are in protected areas, the conservation *of A.*

angustifolia depends heavily on the maintenance of this biome on private lands. Furthermore, an evaluation of the sustainability of natural resource exploitation and land use practices in these properties is necessary, with the goal of integrating them into regional conservation plans (Reis et al. 2012). Planting and restoration of areas in which the species occurs (Vibrans et al. 2012b), for example, should take into account the possibility of establishing areas of seed collection with the potential to obtain high genetic diversity in seed lots, favoring the adaptation of new planted populations (Reis et al. 2012; Vibrans et al. 2012b). In this context, seed collection for seedlings plantation of *A. angustifolia* in grasslands can help to recuperate this threatened species. Conserved grasslands can support the movement of new alleles into continuous forests, acting as a connection between forest fragment edges and functioning as gene flow corridors via pollen and seeds. In the present scenario of high fragmentation and limited connection between fragments (Ribeiro et al. 2009), grassland patches may be an important allelic source for the species in Southern Brazil.

Open environments, such as natural grasslands (Henderson et al. 2016), caatinga, tundra, and savanna (Marinho et al. 2016), have been exploited for many years, whether for pastures or plantations, endangering several species and important biomes worldwide, and these processes are still being supported through public policies. Our results show that open environments such as the grassland, although not considered important for species conservation (Borges et al. 2016), does play a role in the maintenance of genetic diversity for key species of the Mixed Ombrophilous Forest.

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Capítulo 2

Araucaria angustifolia in Natural grassland: dispersal by pollen and seeds in two cohorts

Abstract

Grasslands occur naturally worldwide being one of the largest ecosystems in the world and are under threat of degradation through human activities. The grasslands has been exploited for many years, endangering several species and not considered important for species conservation. Specifically the South Brazilian grasslands occupy some 13.7 million ha and support very high levels of biodiversity and nevertheless they are not protected under the conservation policies. In Brazil, the threatened *Araucaria. angustifolia*, occur in forest and grassland (‘campo’) landscape. Logging and expansion of agricultural land use reduced the forest cover and only 0.62% of the forest is within protected areas and virtually no natural grasslands are protected by law. Previous studies revealed lower genetic diversity and higher inbreeding in younger than older cohorts such as low gene flow, threatening the species. Since dispersal is essential for species to survive, we analyzed the genetic diversity and the dispersal by pollen and seeds of three cohorts (adults, juveniles and progenies) in an entire conserved grassland (‘campo’) area (70 ha) in southern Brazil to answer these issues: 1. Does the species in the natural grassland increase the genetic diversity in recent generations as well as in forests? 2. Is gene flow capable of maintaining genetic diversity in the grassland? Is there a contribution of alleles migrating from the continuous forest around the grassland?

To answer these questions we analyzed all adults and 750 progenies with 9 microsatellites markers from a natural population in a *Campos* grassland area within a conservation area. We concluded the species successfully maintain the genetic diversity and decrease the fixation index. The results showed high gene flow by pollen and seeds and pollen flow between isolated trees, showing an efficient gene flow in open areas. Then, it can suggest tree planting in open areas by degradation to connect forest fragments. The gene flow is long-distances and connecting to continuous forest, being an important landscape and should be created more protected areas by law.

Keywords: *Araucaria angustifolia*, nuclear microsatellites, pollen flow, seed flow, conservation genetics

INTRODUCTION

Grasslands occur naturally worldwide and are one of the largest ecosystems in the world, making up about 40.5% of the earth's terrestrial surface (Suttie et al. 2005). Throughout the world, grasslands are under threat of degradation through human activities (Liu et al. 2017; Saar et al. 2017; Schmid et al. 2017; Zhang et al. 2017) and the degradation is affecting their ability to function properly and is hindering the ability of grasslands to provide the full suite of ecological services they could offer and the causes of degradation are many and may vary by region (Wick et al. 2016). The grasslands have been affected by invasion of species from nearby eucalyptus and pine plantations (Cordero et al. 2016), planted pastures (Grau et al. 2015), agriculture and cultivation of exotic species (Henderson et al. 2016) and livestock overgrazing (Wick et al. 2016).

Open environments, such as natural grasslands (Henderson et al. 2016), caatinga, tundra, and savanna (Marinho et al. 2016), have been exploited for many years, endangering several species and these processes are still being supported through public policies and not considered important for species conservation (Borges et al. 2016).

Campos grassland with patches ('Capoes') in southern Brazil, also known as *Planalto,* are dominate by herbaceous clusters of Gramineae, Cyperaceae, Compost, Leguminosae and Verbenaceae as well as riparian forest and gallery forests modeling the pine forests in the midst of *Campos* grassland formation (Klein, 1978) . Patches ('capoes') are natural formations of forest islands inside de *Campos* grasslands, distinguished by the variable shape and architecture of the edges (Mattos, 1994).

Specifically the South Brazilian *Campos* grasslands occupy some 13.7 million ha and support very high levels of biodiversity and despite their high species richness, the grasslands are not adequately protected under the conservation policies (Overbeck et al. 2007). In the past three decades, approximately 25% of the grassland area has been lost due to land use changes, however, representation of grasslands in conservation units is extremely low (less than 0.5%) and the management in most of these is inadequate to preserve the grasslands (Overbeck et al. 2007).

In Brazil, plant species, such as the threatened *A. angustifolia*, can occur in different types of landscapes, including patches in the natural grasslands (Mattos 1994). The species is a dioecious conifer from South America and it has anemophilous dispersion. Until the early twentieth century, the species occurred across a vast range of approximately 200,000 km^2 in the South and Southeast of Brazil and northeastern Argentina (Reitz and Klein 1966; Hueck 1972; Mattos 1994). Since the Last Glacial Maximum (LGM), the range of the species expanded from valleys to a large areas of natural grasslands in Southern Brazil, favored by warmer climates in the late Holocene (Behling et al. 2009). Furthermore, over the last millennium, range expansion of the species occurred as a result of human groups (Bitencourt and Krauspenhar 2006; Pillar et al. 2009a) for which the tree is a strong cultural symbol, who have played a key role in its rapid territorial expansion (Reis et al. 2014). As a result, the range of the species now includes dense forests and natural grasslands with patches dominated by *A. angustifolia* (Mattos 1994). However, due to uncontrolled and unsustainable exploitation of the Araucaria Forest in Southern Brazil (Guerra et al. 2002) the species has become critically endangered (Thomas 2013). In addition, logging and the expansion of agricultural land use throughout the twentieth century reduced the forest cover to 12.6% of its original area and only 0.62% of the natural range of the forest is currently within protected areas and virtually no natural grasslands are protected by law (Ribeiro et al. 2009).

A. araucaria genetic studies have been revealed lower genetic diversity and higher inbreeding in younger than older generations

(Mantovani et al. 2006b; Bittencourt and Sebbenn 2007; Sant'Anna et al. 2013; Medina-Macedo et al. 2015), such as low gene flow, indicating inefficient maintenance of genetic diversity, increasing the threat of the species (Bittencourt and Sebbenn 2007; Bittencourt and Sebbenn 2008a; Sant'Anna et al. 2013) since dispersal is essential for species to survive the threats of habitat destruction and climate change (Auffret et al. 2017).

The species has never been genetically studied before in the threatened natural grassland landscape inside the conservation area, only in a anthropized area (Cristofolini 2013). So, our main issues are: 1. Does the species in the natural grassland increase the genetic diversity in recent generations as well as in forests? 2. Is gene flow capable of maintaining genetic diversity in the grassland? Is there a contribution of alleles migrating from the continuous forest around the grassland? To answer these questions, we analyzed the genetic diversity and gene flow by pollen and seeds of 3 cohorts (adults, juveniles and progenies) in an entire grassland area in southern Brazil.

Material and Methods

Study site

The study was conducted in a conservation area in Rio Grande do Sul State, Southern Brazil: Centro de pesquisas e conservação da natureza Pró-mata (center for research and nature conservation—Pró-Mata) in the city of São Francisco de Paula (Figure 1). The study area is mostly covered by primary and secondary forests protected and is composed of two natural grassland areas. The area was established in 1996 with an area of 7,660 ha and a previous history of intense logging and fire. Altitudes ranging from 600 to 900 m (Oliveira and Pillar 2005), with an average annual temperature 20°C and rainfall above 2,000mm.

We delimited a grassland area (705.906 m^2) which included different size patches and collected needles from all *A. angustifolia* adults within the established area, for a total sample of 159 adults (65 males and 94 females) (Figure 2). For analysis of the progenies, we collected 30 seeds from 25 seed trees sampled during reproductive events in 2014. We plotted three different areas (900, 3,000 e 16,200m^2) to analyze juveniles individuals (less than or equal 0.3 meters of height) within the patches.

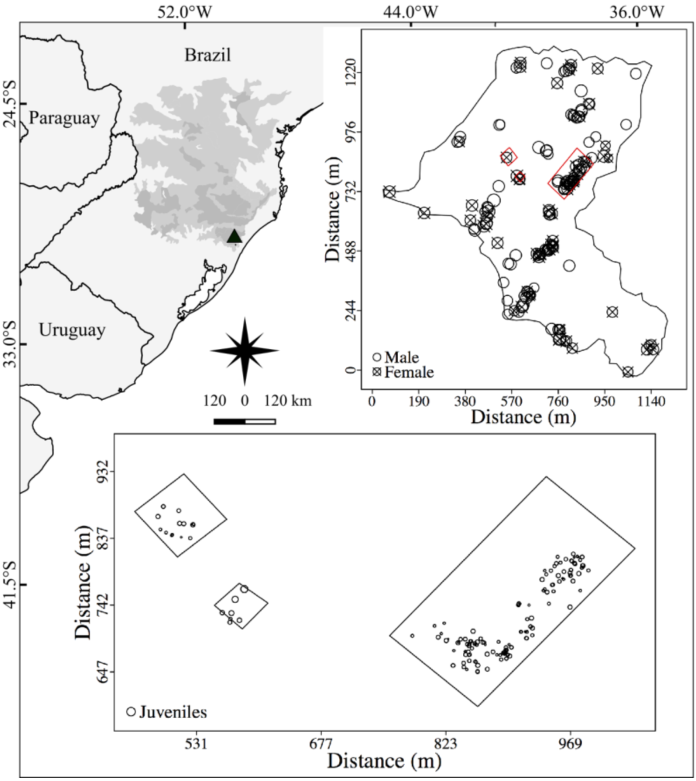

Fig. 4 Geographical distribution of Araucaria Forest (light gray) and the occurrence of areas of the grassland landscape (dark gray) (IBGE 2004). Study site (São Francisco de Paula) in the state of Rio Grande do Sul in Southern Brazil indicated by triangle and the distribution of reproductive trees of *Araucaria angustifolia* (o = males, x = females). Total grassland

area (705.906 m^2) and three plots (900, 3,000 e 16,200m^2) for with juveniles generation located in Atlantic Rainforest, South of Brazil.

Extraction and amplification of DNA

DNA was extracted from the embryo of the seeds, needles of adult trees and juveniles with the NucleoSpin Kit Plant II® (Macherey-Nagel). For DNA amplification, we used nine microsatellite loci in two multiplex system (Table 5). The reaction had a total volume of 5 ul containing 2.5 KAPPA2G, 0.2 mM of forward primer, 0.2 mM of reverse primer, 0.5 ul of water and 10 ng DNA. Cycles were 95 °C for 3 minutes followed by 35 cycles consisting of three steps: the first at 95 °C for 30 seconds; the second at T (a) °C for 45 seconds; and the third at 72 °C for 1 minute, with a final extension at 72 °C for 10 minutes. We genotyped in an Automated Genetic Analyzer ABI 3500xL (Applied Biosystems) using the capillary size standard LIZ GS 600. Data were analyzed based on the interpretation of electropherograms using the GeneMapper software (Applied Biosystems).

Table 5. Characteristics of microsatellite markers used for grassland population of *Araucaria angustifolia*. Ta, annealing temperature (° C); R, expected size range in base pairs (bp).

Primers	Fluorochrome	Repeat motif	T_a	R (bp)	Reference
Ag20	FAM	$(GA)_{12}$	60	257-283	Salgueiro et al. 2005
Ag23	NED	$(TA)_5(GT)_4$	60	249-287	Salgueiro et al. 2005
Ag45	FAM	$(GT)_4AT(GT)_7$	60	170-186	Salgueiro et al. 2005
Ag56	PET	$(TC)_{11}$	60	132-180	Salgueiro et al. 2005
Ag94	FAM	$(TC)_{12}$	56	159-203	Salgueiro et al. 2005
CRCA1	FAM	$(GA)_{19}$	56	212-232	Scott et al. 2003
CRCA2	VIC	$(GA)_{23}$	56	199-219	Scott et al. 2003
Aang22	FAM	$(GA)_{10}$	56	220-250	Schmidt et al. 2007
Aang24	NED	$(CT)_{19}$	56	160-200	Schmidt et al. 2007

Prior Genetic analysis

Before including the microsatellite set in our study, we tested for gametic disequilibrium and presence of null alleles. As gametic disequilibrium creates pseudo-replication for analyses in which loci are assumed to be independent samples of the genome, we used the F_{STAT} 2.9.3.2 software (Goudet 2002) to test all loci for linkage disequilibrium, applying the Bonferroni correction for multiple comparisons (Rice 1989). Null allele frequencies were assessed for the populations using the Microchecker 2.2.3 software (Van Oosterhout et al. 2004). If significant homozygosity was detected at a given locus, it was excluded and a modified average inbreeding coefficient (F_{IS}) was calculated across all loci; its significance was estimated using a jackknife across all loci.

Genetic diversity analysis

Genetic diversity indices were averaged across all loci for all sampled adults, juveniles and progenies. The estimated parameters were: average number of alleles per locus, A; allelic richness, *AR*; observed heterozygosity, H_o; and expected heterozygosity, H_e. To infer the levels of inbreeding, we estimated the inbreeding coefficient (F_{IS}) and Nason's estimator of coancestry coefficient (F_{ij}), described in Loiselle et al. (1995), for each population. Significance (determined by 10,000 permutations across loci) was tested using the SPAGeDi program (Hardy and Vekemans 2002). All analyses and estimates were carried out using the F_{STAT} program (Goudet 2002).

Paternity analysis and gene flow by pollen

Paternity and maternity analysis was assigned by comparing the genotypes of adult males, females and seeds using the program CERVUS 3.0 (Kalinowski et al. 2007). The relationship was determined by Δ statistic, which is the difference between the LOD (logarithm likelihood ratio) of the most likely candidate parent minus the LOD of the second most likely candidate parent. To find the critical value of Δ (the difference in LOD scores between the two most likely parents), indicating the confidence level of paternity analysis, simulations were conducted, using 10,000 repetitions, 0.01 as the proportion of misclassified loci, and all individuals as probable father candidates for each mother tree. We used 95% as the strict confidence level and 80% as the relaxed, as suggested by Marshall et al. (1998). From the paternity analysis, pollen dispersal distance was calculated based on the position of the seed tree and the putative pollen parent within the study plots and from the maternity analysis, seed dispersal distance was calculated based on the position of the seed tree and the juvenile within the study plots.

Determination of the mating system

The mating system was analyzed under the mixed-mating and correlated mating models, using the Multilocus mating system software MLTR, version 3.2 (Ritland 2002). The parameters estimated were: single-locus outcrossing rate ($\hat{t}_s$); multilocus outcrossing rate ($\hat{t}_m$); selfing correlation ($\hat{r}_s$); biparental inbreeding rate ($\hat{t}_m - \hat{t}_s$); multilocus paternity correlation ($\hat{r}_{p(m)}$) and effective number of pollen donors. To determine whether the values were significantly lower than 1 ($\hat{t}_m - \hat{t}_s$) or greater than 0 ($\hat{t}_m - \hat{t}_s$, and $\hat{r}_{p(m)}$), a 95% confidence interval was calculated.

Results

Genetic analysis

For the genotypic disequilibrium analysis, none of the loci pair combinations showed significant deviation ($p < 0.001$) after Bonferroni correction. Thus, the loci could be used for all genetic analyses. We found significant frequencies of null alleles for adults, juveniles and progenies.

We found a total of 87 alleles for adults, 81 for juveniles and 107 for progenies. The expected heterozygosity for grassland adults, juveniles and progenies was 0.68, 0.67 and 0.65, respectively, and the observed heterozygosity was 0.57, 0.59 and 0.58 (Table 6). The values of F_{IS} were significant and different from zero for both (Table 6). The allelic richness was higher for progenies (11.6) than adults (9.44) and juveniles (8.81).

Table 6. Genetic diversity indices and inbreeding coefficient in the population of grassland for three generations (adults, juveniles and progenies) of *Araucaria angustifolia*. n is the sample size, AR the allelic richness, He the average expected heterozygosity in Hardy-Weinberg equilibrium (CI: confidence interval calculated by 1000 bootstraps), Ho the average observed heterozygosity (CI: confidence interval calculated by 1000 bootstraps), and F_{IS} is the fixation index (* $p<0,05$).

	Grassland São Francisco de Paula				
	n	AR	He	Ho	F_{is}
Adults	159	9.44	0.68(0.67-0.68)	0.57(0.55-0.60)	0.15*
Juveniles	149	8.81	0.67(0.66-0.68)	0.59(0.56-0.62)	0.13*
Progenies	750	11.6	0.65(0.65-0.66)	0.58(0.55-0.58)	0.11*
Average			0.66	0.58	0.13*

Paternity and maternity assignment and gene flow by pollen and seeds

In this population, the pollen dispersal distance obtained through paternity analysis ranged from 2 to 1,226 m with an average of 482 m for progenies analysis and 2 to 1,037 m with an average of 415m for juveniles analysis (figure 2). We found 669 progenies with 59 different fathers in the area and 11% are from migrant pollen (81 progenies). For juveniles we found 48 fathers and 15 from migrant pollen. The seed dispersal ranged 1 to 918 m with an average of 229 m (figure 2). We found 59 different seed trees and (11%) from migrant seeds (16 juveniles), the same for migrant pollen.

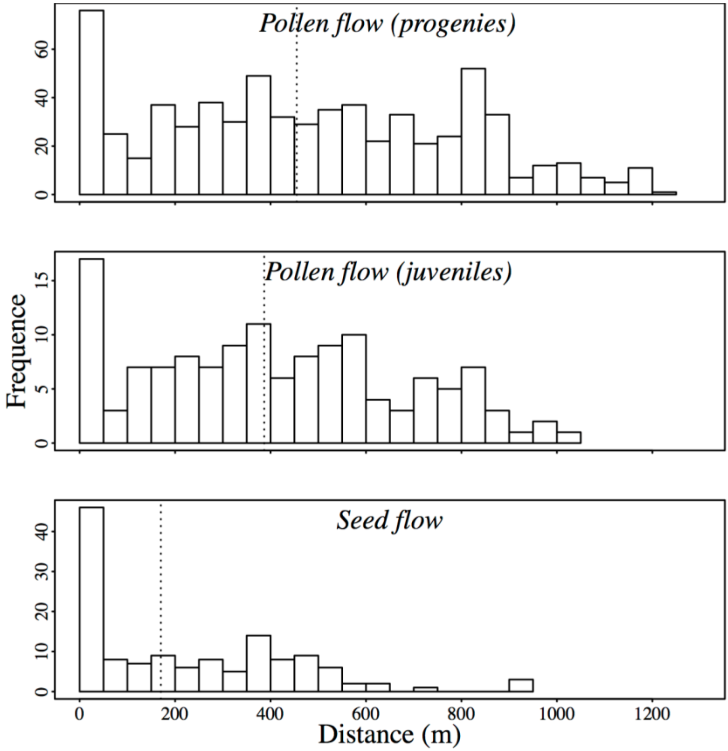

Fig. 5 Frequency of pollen dispersal distances by progenies and juveniles analysis and frequency of seed dispersal in the natural grassland population (*São Francisco de Paula*) of *Araucaria angustifolia*.

Mating system

For the population, the multilocus outcrossing rate ($\hat{t}_m$) was 0.998 and single locus outcrossing rate ($\hat{ts}$) was 0.951. The difference between these rates is low, but significantly different from zero for grassland ($\hat{t}_m$ - $\hat{t}_s$ = 0.047), suggesting a limited rate of mating among relatives. The $\hat{r}_{p(m)}$ value indicates that more than 10 fathers ($\hat{N}_{ep}$ = 10.52) contributed to individual progeny arrays in the natural grassland population (table 7).

Table 7. Mating system parameters of *Araucaria angustifolia* in natural grassland in the Atlantic Rainforest, Santa Catarina State, Southern Brazil.

Mating system	
Population	Grassland
Multilocus outcrossing rate	0.998
Single-locus outcrossing rate	0.951
Selfing rate	0
Mating among relatives	0.047
Correlation of selfing	-0.999
Multilocus paternity correlation	0.095
Effective number of pollen donors	10.52

Discussion

The results revealed an efficient maintenance of genetic diversity in the natural grassland landscape with a trend to increase the observed heterozygosity for progenies and juveniles than adults, even as increasing of inbreeding. These values are lower in others forest studies for younger generations for *A. angustifolia* (Mantovani et al. 2006b); (Bittencourt and Sebbenn 2007); (Sant'Anna et al. 2013); (Medina-Macedo et al. 2015), *A. nemorosa* (Kettle et al. 2007b), *Hymenaea courbaril* (Carneiro et al. 2011), *Copaifera langsdorffii* (Sebbenn et al. 2011a) and *Vateriopsis seychellarum* (Finger et al. 2012).

The population has only 1.34 ind.ha^{-1} of females contrasting to density of reproductive females in forests landscapes with 40 ind.ha^{-1}, 13.9 ind.ha^{-1} and 18 ind.ha^{-1} reported for forest reserves in Santa Catarina and São Paulo (Mantovani et al. 2006b); (Cristofolini, 2013); (Paludo et al. 2009b). One species characteristic is a higher percentage of males than females (Mattos 1994) and we found more females than males (1.4:1) in this natural grassland, as well as different of the grassland in a particular area (1:1.6) with more males (Cristofolini, 2013). Since most pollen travels limited distances in wind-pollinated plants, both the local quantity and diversity of mates may limit female reproductive success (Vanderpitte et al. 2009).

Nevertheless unlike the expected with lower number of males in the grassland, the pollen flow through the progenies analysis was high and efficient to maintain the genetic diversity and increase the inbreeding with an average of 482 meters of distance for pollen flow and 81 progenies pollinated from continuous forest, outside the grassland. The progenies generation was greater than the older generation (juveniles). The analysis showed the similar success with 415 meters of distance and 15 juveniles from migrant pollen.

The outcrossing rate presented with reduced mating among relatives (0.047) as well as a high number of fathers in this population, corroborate to results about pollen flow favored by open area.

A. angustifolia in continuous forests showed the average pollination distance 102 and 134 meters, respectively, however a high proportion in short-distance producing biparental and correlated mating as well as reducing the variance effective size (Bittencourt and Sebbenn 2008a; Sant’Anna et al. 2013). The species has normally high population densities in forests, which may limit the movement of pollen between and within populations and reduce the number of pollen donors contributing to the next generation (Sousa and Hattemer 2003). Low levels of pollen immigration and short-distance of pollen dispersal, associated to spatial genetic structure in forests, can reduce genetic diversity and variance effective size within progenies (Bittencourt and Sebbenn 2008). Thus, the open-area grassland characteristic is positive for pollen flow, nevertheless have been affected by invasion of species from nearby eucalyptus and pine plantations (Cordero et al. 2016), planted pastures (Grau et al. 2015), agriculture and cultivation of exotic species (Henderson et al. 2016) between the Araucaria patches. For example, Costa et al. (2015) reported that patches of *A. angustifolia* interspersed among *Pinus* plantations showed fixation indexes from inbreeding which differ from areas not surrounded by plantations, indicating barriers to pollen flow and decreased migration of alleles between patches. And our results showed high gene flow by pollen and seeds and pollen flow between isolated trees, showing an efficient gene flow in open areas. Then, it can suggest tree planting in open areas by degradation to connect forest fragments.

Other important issue for gene flow success is the seed dispersal. The results in the natural grassland landscape revealed long-distance seed dispersal (1 to 918m) , average (229m) and migrant seeds higher than studies within forests with average 131m and 5% of migration (Sant’Anna et al. 2013). Seed dispersal in a small forest fragment of *A. angustifolia* ranged from 0.35 to 291 m, with 47 % of seeds dispersed effectively within a radius of 60 m around the mother tree (Bittencourt and Sebbenn 2007). Previous studies for *A. angustifolia* and other species suggest a high intensity of short-distance seed dispersal for species with gravity dispersed seeds. For *Quercus salicina* in 11.56 ha just 15% of immigration seeds (Nakanishi et al. 2009) and *Fagus crenata* a maximum seed dispersal distance of 20 m and 30 m for two plots (Asuka et al. 2005). In our results the seed dispersal was higher showing almost 70% over 100m and 11% of migrant seeds from forest and juveniles migrating from different patches and isolated trees within the 70 ha of natural grassland.

A previous study showed the presence of the main Araucaria dispersers in the area, the mammals *Dasyprocta azarae* (agoutis), *Cuniculus paca* (pacas) and the birds *Amazona pretrei* (red-spectacled parrot) and *Cyanocorax caeruleus* (blue jay) in the same conservation area (Iob and Vieira 2008) that can explain the greater seed dispersal distance in this grassland. The same species have not found in the grassland in a particular area with grazing, only the blue jay (Schüssler 2014). Even despite the importance of seed dispersal for survival of plant species, data on seed dispersal at landscape scales remain sparse and effective seed dispersal among fragments determines re-colonization and plant species persistence in such landscapes (Ismail et al. 2017).

The efficient allelic exchange within the grassland and between isolated trees and the continuous forest around in this work and previous work in an anthropized area (Cristofolini 2013) shows both the importance of keeping this landscape protected in conservation areas, as well as in private properties, because the vast majority of remaining forest fragments in Southern Brazil are found on small farms. Thus, it is necessary to give incentives for landowners to maintain and sustainably use these areas. Likewise create more conservation areas in different regions is also necessary, especially to protect the existing fauna such as raise public awareness of the value and vulnerability of the grassland.

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Capítulo 3

Considerações e perspectivas de conservação para campo e floresta

Esta tese teve por objetivo compreender a dinâmica da diversidade genética em populações da espécie *Araucaria angustifolia* em paisagem de campo e floresta no Sul do Brasil, abordando variações no sistema reprodutivo, diversidade genética e elementos de fluxo gênico. Assim, buscou gerar avanços na discussão de estratégias de conservação e uso da espécie nestas paisagens distintas evolutivamente e historicamente exploradas para uso da madeira, plantio de espécies exóticas e pastejo.

Dentre os principais resultados, a população da Floresta Nacional de Três Barras apresentou tendência a maior diversidade genética em adultos do que em progênies, o contrário do que foi encontrado tanto na população de campo em unidade de conservação como quando comparada com a população de campo antropizado, o que sugere uma eficiência na manutenção da diversidade genética na paisagem de campo. Da mesma forma, a população de campo em unidade de conservação apresentou a uma tendência ao aumento de diversidade genética tanto para as progênies como para a coorte dos juvenis. Para as três populações o índice de fixação foi reduzido da coorte de adultos para a coorte das progênies, incluindo a coorte de juvenis na população de campo em unidade de conservação. Porém, para a população de campo antropizado, o índice foi reduzido a 0,03, não sendo significativo, o que também indica uma maior eficiência em relação a paisagem de floresta.

A dispersão do pólen dentro da floresta foi a menores distâncias (de 9 a 206m com média de 91m) do que no campo antropizado (de 10 a 334m com média de 126m). Já a dispersão de pólen para a população de campo em unidade de conservação foi realizada a distâncias ainda maiores, tanto avaliada através da distância entre os pais e as progênies (de 2 a 1.226m com média de 482m), quando através da distância entre os pais e mães dos indivíduos juvenis (de 2 a 1.037m com média de 415m), representando também maior eficiência no fluxo de pólen nas gerações mais recentes no campo, assim como observado em relação a diversidade genética.

Nas três populações a taxa de cruzamento não foi diferente de 1,0 e o número de pais foi maior na população de floresta (23,80) do que na população de campo antropizado (16,39) e na população de campo em unidade de conservação (10,52), possivelmente pelo fato de a primeira população apresentar maior número de indivíduos machos

(163), a segunda (93) e a terceira com menor número (65). Mesmo com estas diferenças em densidade, as populações de campo apresentaram maiores índices de diversidade genética e menores índices endogâmicos.

A dispersão de sementes foi a longas distâncias (de 1 a 918m com média de 229m) e com uma taxa de migração de 11% vinda da floresta que circunda o campo natural da unidade de conservação, na qual estudos anteriores mostram que estão presentes os principais conhecidos dispersores da espécie. Na população da Floresta Nacional de Três Barras, apesar da presença dos dispersores da espécie assim como de predadores, não foram encontrados indivíduos na coorte de juvenis para ser realizada esta análise de dispersão de sementes.

Todos os resultados apresentados nesta tese mostram que as três populações apresentam potencial para conservação da espécie se devidamente geridas. Na Floresta Nacional de Três Barras a população estudada é composta principalmente por indivíduos antigos, os quais não aumentam a diversidade genética das progênies, reflexo de uma intensa exploração madeireira na área. Já as populações de campo em propriedade particular e em unidade de conservação mostraram resultados ímpares, principalmente em relação ao fluxo gênico a maiores distâncias, eficiente para manutenção da diversidade genética.

A paisagem de campo mostrou uma conexão importante com a floresta, principalmente na população em unidade de conservação, na qual a fauna tem o transito facilitado por não haver presença de gado. Assim como os resultados de fluxo de pólen nos dois campos, ocorrendo entre capões e árvores isoladas em modelo de “trampolim” de trocas alélicas chegando até a floresta contínua. O modelo de fluxo gênico em “trampolim” em ambientes abertos expostos a ventos avaliado neste trabalho pode ser aplicado na recuperação de florestas da espécie, conectando pequenos fragmentos isolados através da **implantação/manutenção de árvores isoladas ou conjunto de árvores.** Assim como mostram estudos em ambientes abertos de florestas degradadas indicando fluxo de pólen mais efetivo nas áreas abertas e entre árvores das bordas de florestas, como discutido por (Medina-Macedo et al. 2015) e dispersão de pólen a maiores distâncias entre grupo de árvores isoladas do que dentro da floresta, realizando a conexão entre fragmento e floresta O mesmo acontece para *Araucaria araucana*, em ambiente naturalmente aberto, semelhante à paisagem de campo, com maiores distâncias de dispersão de pólen (90 a 10.000 m) do que a população florestal contínua (17,42 a 5.163 m) (Moreno 2012). Outras espécies como, o endêmico carvalho vermelho, indicam um fluxo de pólen extenso entre fragmentos e árvores isoladas, conferindo-lhes um grande valor para a conservação da diversidade genética e conectividade (Oyama et al. 2017). O mesmo ocorre com uma espécie ameaçada de extinção (*Gomortega keule*) na qual o pólen se deslocou de pequenas populações e de árvores individuais para grandes populações, assim como na direção contrária, indicando que estas populações e árvores individuais tem um papel chave na população maior e vice-versa, servindo como trampolins de fluxo gênico (Lander et al. 2010). Portanto, analisando o fluxo de pólen das espécies o modelo de implantação de árvores isoladas ou conjuntos de árvores isoladas para conexão de fragmentos parece ser eficiente para muitas outras espécies também.

A problemática do ambiente aberto, especialmente nos campos naturais, é principalmente a agricultura e cultivo de espécies exóticas (Costa et al. 2015; Henderson et al. 2016), invasão/implementação de espécies exóticas como espécies de eucaliptos e pinheiros dentro do campo (Cordero et al. 2016) e plantação de pastagens (Grau et al. 2015), resultando em barreiras para o fluxo gênico, além da perda da riqueza de espécies nativas (Cordero et al. 2016), importantes para o ecossistema e fauna. Em estudo anterior em propriedade particular em campos naturais, Costa et al. (2015) relataram que capãos de *A. angustifolia* intercalados entre plantações de *Pinus* apresentaram índices de fixação de endogamia que diferem de áreas não cercadas por plantações, indicando barreiras ao fluxo de pólen e diminuição da migração de alelos entre as manchas. Esses resultados reforçam a importância do desenvolvimento de estratégias de conservação e estratégias alternativas de uso do solo nesses ambientes, como o **reflorestamento com *A. angustifolia***, e a exploração parcial para gerar lucros em áreas particulares. Além disso, a substituição de plantações de árvores exóticas por plantações de *A. angustifolia* nativas (Vibrans et al. 2012a), se devidamente geridas, poderia reduzir gradualmente o número de propagulas de espécies invasoras em paisagens de campo (Barbosa et al. 2009; Cordero et al. 2016). As plantações de *A. angustifolia* têm um alto potencial como locais de colonização por espécies lenhosas nativas, mesmo com diferenças estruturais em relação às florestas nativas (Mendonça-Lima et al. 2014) e também atua como uma eficaz espécie nucleadora (*nurse*) que nutre o desenvolvimento de mudas (Korndörfer et al. 2015) como também já observado em estudo de nucleação em matriz de campo dos planaltos de alta altitude no Brasil (Dos Santos et al. 2011), tornando-se um caminho positivo para a recuperação de áreas degradadas.

Outra sugestão que poderia minimizar impactos como os de plantações de espécies exóticas de longa dispersão de pólen como o *Pinus* sp, seria cercar essas plantações com *A. Angustifolia*, criando uma barreira natural para reduzir a dispersão de propágulos da espécie exótica e aumentando as possibilidades de trocas alélicas para Araucária.

Considerando os resultados de elevado fluxo de pólen no campo antropizado em área privada, especialmente a redução do índice de fixação a não significativo na coorte das progenies recomenda-se fortemente a **conservação e uso sustentável em áreas privadas.** Além disso, a grande maioria dos fragmentos florestais remanescentes no sul do Brasil é encontrada em pequenas fazendas, portanto é crucial incentivar proprietários a manter e utilizar de forma sustentável.

Além disso, é necessária uma avaliação da sustentabilidade da exploração de recursos naturais e práticas de uso do solo nessas propriedades, com o objetivo de integrá-las em **planos regionais de conservação** (Reis et al. 2012). A plantação e restauração de áreas em que a espécie ocorre (Vibrans et al. 2012b), por exemplo, deve levar em conta a possibilidade de estabelecer áreas de coleta de sementes com potencial para obter alta diversidade genética em lotes de sementes, favorecendo a adaptação de novas populações plantadas (Reis et al. 2012; Vibrans et al. 2012b). Neste contexto, **a coleta de sementes para produção de mudas** de *A. angustifolia* em campos onde o fluxo gênico mostrou-se mais eficiência deve ser feita, assim como a coleta local de sementes em áreas abertas em ambiente florestal, nas quais o fluxo gênico também é facilitado para produção de mudas para recuperação e reflorestamento. Além disso, os campos conservados podem apoiar o movimento de novos alelos para as florestas contínuas, atuando como uma conexão entre as bordas dos fragmentos florestais e funcionando como corredores de fluxo genético via pólen e sementes.

Apesar de escassos em escala de paisagem dados de dispersão de sementes são importantes para avaliação de estratégias de conservação, pois determinam a re-colonização e a persistência de espécies de plantas em tais paisagens (Ismail et al. 2017). Estudos anteriores para *A. angustifolia* e outras espécies sugerem uma alta intensidade de dispersão de sementes de curta distância para espécies com sementes dispersas pela gravidade, portanto torna-se importante a conexão entre fragmentos também para o trânsito da fauna dispersora. Nos resultados desta tese a dispersão de sementes mostrou quase 70% acima de 100m e 11% de sementes migrando da floresta e de diferentes capões e árvores isoladas dentro dos 70 ha de campo natural na unidade de conservação estudada, atribuida a fauna dispersora da espécie presente na área. Portanto a **criação de mais unidades de conservação** em diferentes regiões e tipo de campos naturais também é de grande importância para sobrevivência e atuação da fauna na dispersão da espécie, assim como a conscientização pública sobre esta paisagem.

Finalmente, a espécie *Araucaria angustifolia* hoje criticamente ameaçada de extinção necessita de sua existência nas duas paisagens evolutivamente e historicamente diferentes de ocorrência natural, as quais fazem parte uma da outra, mostrando um eficiente mosaico de trocas alélicas via pólen e via sementes quando protegidas. E mais que isso, a paisagem de campo natural revelou um fluxo gênico como ainda não foi visto em paisagem florestal. Portanto, no cenário atual de alta fragmentação e limitada ligação entre fragmentos, a espécie no campo natural é uma fonte alélica importante e uma paisagem chave para a sua ameaça no sul do Brasil.

ANEXOS

Metodologias de laboratório

Amplificação do DNA:

Os dois sistemas multiplex utilizados para amplificação do DNA com os nove marcadores microssatélites utilizados no estudo:

Multiplex 1		**Multiplex 2**	
KAPPA2G	2,5 ul	KAPPA2G	3 ul
H2O	0,5 ul	H2O	0,96 ul
DNA	1 ul	DNA	1 ul
Forward:		Forward:	
AG20	0,15 ul	AG94	0,1 ul
AG23	0,05 ul	CRCA1	0,02 ul
AG45	0,2 ul	CRCA2	0,15 ul
AG56	0,1 ul	Aang22	0,1 ul
Reverse:		Aang24	0,1 ul
AG20	0,15 ul	Reverse:	
AG23	0,05 ul	AG94	0,1 ul
AG45	0,2 ul	CRCA1	0,02 ul
AG56	0,1 ul	CRCA2	0,15 ul
		Aang22	0,1 ul
		Aang24	0,1 ul
Volume total	5 ul	Volume total	5 ul

Ciclos: 95 °C por 3 minutos seguidos de 35 ciclos de três passos: o primeiro a 95 °C por 30 segundos; o segundo a T (a) °C por 45 segundos; e o terceiro a 72 °C por 1 minuto, com a extensão final a 72 °C por 10 minutos.

Preparação do DNA amplificado para corrida no sequenciador ABI3500Xl

Corrida ABI3500xL	
H_2O:PCR	20ul:1ul
PCR diluída	1 ul
LIZ GS 600	0,25 ul
Formamida	8,75 ul
Volume total	10 ul

Figura 1. Parcela em paisagem de campo dentro de propriedade particular na região da Coxilha Rica, em Lages no Estado de Santa Catarina.

Figura 2. Parcela em paisagem florestal dentro da Floresta Nacional de Três Barras, em Três Barras no Estado de Santa Catarina.

Figura 3. Área de campo avaliada e parcelas para avaliação dos juvenis no Centro de pesquisas e conservação da natureza Pró-mata, em São Francisco de Paula, no Estado do Rio Grande do Sul.