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Short Communication

PCR-RFLP analysis of non-coding regions of cpDNA in *Araucaria angustifolia* (Bert.) O. Kuntze

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Abstract

The *Araucaria angustifolia* (Bert.) O. Kuntze, also named the "paraná pine" ('pinheiro-do-Paraná' in Portuguese), is a native conifer species naturally occurring in the Brazilian Tropical Atlantic Forest which in Brazil is mostly limited to the southern Brazilian states of Paraná, Santa Catarina and Rio Grande do Sul. Chloroplast DNA markers (cpDNA) are useful in populational genetic studies because of their low substitution rate and the uniparental transmission. The conservation of cpDNA genes between species has allowed the design of consensus chloroplast primers that have had a great impact on population genetics and phylogenetic studies. In this study we used the polymerase chain reaction technique combined with restriction enzyme fragment length polymorphism (PCR-RFLP) to characterize the genetic diversity of the chloroplast genome in nine natural *A. angustifolia* populations. Among the 141 trees surveyed we found 12 different cpDNA haplotypes and demonstrated that *A. angustifolia* has high levels of total diversity (h_T = 0.612) and an average within-population diversity (h_S) of 0.441, suggesting the presence of high within-population variation. The estimated genetic divergence could be helpful in designing breeding programs and species conservation strategies, although additional studies with a larger number of populations and trees is essential for a better understanding of gene flow and the inheritance of major *Araucaria angustifolia* traits.

Key words: haplotypes, conifer species, Brazilian pine, genetic diversity.

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The tree *Araucaria angustifolia* (Bert.) O. Kuntze, popularly named 'pinheiro-do-Paraná' (the pine from Paraná state) is a conifer species native to tropical Atlantic forest and, in Brazil, can be found mainly in the three southernmost states of Paraná, Santa Catarina and Rio Grande do Sul. This species has significant ecological, economic and social values and the seeds of *A. angustifolia* are an important food source for fauna, including the birds and rodents, which are the main means of dispersion for this species. Besides being a dominant tree, populations of adult araucaria create a microcosmic environment where shade-tolerant plant species of other taxa can grow and develop (Auler *et al.*, 2002).

Before the arrival of Europeans around 500 years ago, the araucaria forest has been estimated to have covered about 200,000 km^2 (Seitz, 1986) but the exploitation of araucaria due to the high quality of its wood and the spread of agricultural has resulted in *A. angustifolia* being given vulnerable category status (IBAMA, 1992) as defined by the International Union for the Conservation of Nature and Natural Resources Red Book (IUCN, 2004). However, since most of the remaining araucaria, amounting to 1 to 3% of the original area, are still under pressure from uncontrolled exploitation by the timber industry (Auler *et al.*, 2002) conservation and sustainable management strategies should be implemented as soon as possible. It thus follows that knowledge of the genetic structure of the remaining araucaria populations is crucial to defining strategies to ensure the proper conservation and sustainable use of the natural araucaria ecosystems.

Since the development and utilization of molecular markers the genetic structure of natural populations has been studied intensely and these studies have characterized how genetic variation is distributed within and between populations and which factors determining the genetic

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structure of a natural population. Szmidt and Wang (1994) having pointed out that such information could help define conservation and management strategies.

In araucaria, few studies have been carried out with molecular markers, although Stefenon *et al.* (2004) have reported that, in comparison to allozymes, RAPD markers are able to associate lower genetic similarities with large geographical distances between *A. angustifolia* populations and detected a higher level of polymorphism. In comparison to the study by Stefenon *et al.* (2004), Auler *et al.* (2002) detected a lower level of genetic variation in nine natural *A. angustifolia* populations in Santa Catarina, and also found that the diversity indexes were lower in degraded *A. angustifolia* populations than in better-conserved populations.

Nevertheless, there has been a growing interest in the use of chloroplast DNA (cpDNA) in the populational genetic studies of plants. Indeed, despite its low substitution rate (Wolfe *et al.*, 1987) and typically uniparental transmission (Hipkins, 1994), the clonal mode of evolution is a unique feature of cytoplasmic genomes which is of great interest in genetic studies (Pons and Petit, 1995). Several universal primers for amplifying non-coding spacers of the chloroplast genome have been reported (Taberlet *et al.*, 1991; Demesure *et al.*, 1995; Dumolin-Lapègue *et al.*, 1997). Consensus primers, which are homologous to the most conserved coding regions but amplify variable non-coding regions, are very useful in phylogenetic and populational studies (Taberlet *et al.*, 1991; Demesure *et al.*, 1995; Dumolin-Lapègue *et al.*, 1997). In particular, the conservation of the arrangement of the genes in cpDNA has allowed the design of consensus chloroplast primers that have had a great impact on population genetics and phylogenetic studies by sequencing or by PCR – RFLP (Taberlet *et al.*, 1991; Demesure *et al.*, 1995). Some studies using PCR-RFLP have shown higher levels of polymorphism in the chloroplast genome than allozyme loci in populations of *Picea densata* but did not find any differentiation or polymorphism in populations of *Picea tabulaeformis*, *Picea yunnanensis* and *Picea massoniana* (Wang and Szmidt, 1994).

The objective of this study was to test the universal set of cpDNA pair of primers to non-coding region for their ability to characterize genetic polymorphisms in fragmented natural populations of *A. angustifolia* in the Brazilian state of Santa Catarina and their utility in providing support for the development of species conservation, sustainable management and breeding strategies.

Leaf and seed samples were randomly collected from 141 adult specimens from eight natural *A. angustifolia* populations in different regions of Santa Catarina (Figure1, Table 1). The total DNA from leaf, megagametophytes and embryos, both isolated from seeds, were extracted using a

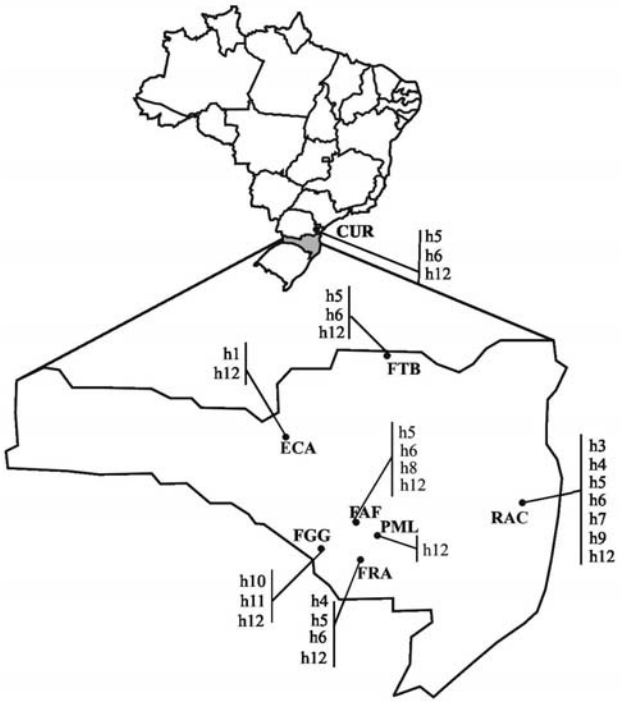

Figure 1 - Geographic location of the sampled populations in Santa Catarina State (Brazil) and the haplotype distribution. PML = Parque Municipal de Lajes, FTB = Flona de Três Barras, FRA = Fazenda Rancho Alegre, ECA = Estação de Caçador, FGG = Fazenda Guamirim Gateados, RAC = Reserva do Caraguatá/Antônio Carlos, FAF = Fazenda Amola Faca. h1-h12 = haplotype names. See Table 1 for Longitude and latitude of each site.

Table 1 - Geographic location, altitude, population size, forest type and history of use of eight *A. angustifolia* populations.

Population code	Population size	Location	Longitude and latitude	Altitude (m)	History of use
FTB	27,000	Três Barras	26°06'23" S, 59°19'20" W	800	Selective cut, secondary forest
FRA	2,700	Lages	27°53'18" S, 50°15'18" W	920	Conserved area
RAC	500	Antônio Carlos	27°25'84" S, 48°50'98" W	600-910	Timber exploitation, grazing, forest reserve
FAF	1,700	Lages	27°48'58" S, 50°19'34" W	920	Selective cut, grazing
FGG	40,000	Campo Belo do Sul	27°53'57" S, 50°43'39" W	960	Wild conserved area
PML	400	Lages	27°47'52" S, 50°22'30" W	920	Very degraded area
ECA	10,000	Caçador	26°46'31" S, 26°46'31" W	920	Conserved since 1950
CUR	> 1,000	Curitiba	25°25'48" S, 49°16'15" W	900	Timber exploitation

previously described method (Ferreira and Grattapaglia, 1995).

The 17 primers used in this study were: TF, AF (Taberlet *et al.*, 1991), K1K2, HK, CD, DT, CS, SfM, AS, ST (Demesure *et al.*, 1995), and FV1, V2L, TC, C1C, K2Q, QR and fMA (Dumolin-Lapègue *et al.*, 1997). The PCR amplifications were carried out in a total volume of 50 µL containing about 50 ng of total DNA, 2 mM of $MgCl_2$, 2 µM of each primer, 10x *Taq* polymerase buffer (Gibco-BRL), 0.5 U of *Taq* polymerase (Gibco-BRL), and 0.25 µM of dNTPs. The PCR products (10 µL) were digested for 2 h using the *Hinf*I, *Hind*III, *Rsa*I, *Hae*III, *Taq*I, *Bam*HI, *Knp*I and *Eco*RI restriction enzymes, each digestion being accomplished in a total volume of 15 µL at the temperatures recommended by the manufacturer of the enzymes (Gibco-BRL, Brazil). The digestion products were separated on 8% (w/v) non-denaturing polyacrylamide gels for 3-4 h at 300 V using a Protean II apparatus (BioRad, US) and the DNA fragments visualized by silver staining. The reproducibility for each PCR and enzyme/PCR product combination was assessed by replicating each reaction. The haplotypes were obtained by the combination of all primer/restriction enzymes and were named h1-h12 (Table 2). The diversity indices, total genetic diversity (h_T), within-population diversity (h_S), and the differentiation index G_{ST} were calculated using the HAPLODIV program developed by a Pons and Petit (1995).

Of the 17 loci tested 11 (HK, CD, ST, K2Q, C1C, TC, FMA, TF, FV1, V2L and AF) failed to produce detectable PCR products in *A. angustifolia* but six yielded distinct PCR products, these loci represented a total of ~14 kb or approximately 10% of the total *A. Angustifolia* chloroplast genome assuming that the size of the chloroplast genome of conifers is 120 kb (Wakasugi *et al.*, 1994). In our study, all the *A. angustifolia* cpDNA PCR products were monomorphic (Figure 2A). Like some other conifer species, the cytoplasmic genome is present in both the megagametophyte and embryo of *A. angustifolia* (Wang *et al.*, 1995) and our research shows that cpDNA non-coding regions can be amplified from both megagametophyte and embryo tissue, suggesting that the chloroplast genome could be used to study inheritance transmitted by both seed and pollen (Figure 2A).

The combinations of primers/enzymes utilized in this study were able to detect different haplotypes within each *Araucaria* population (Table 2, Figure 2B and 2C), finding 12 distinct haplotypes of which 66.7% were population specific and one-third was shared by at least five of the eight *A. angustifolia* populations studied. On average, there were 3.5 haplotypes per population per pair of primers, and the number of within population haplotypes varied from one to seven. The proportion of differentiation between populations (G_{ST}) was 0.280, which is higher than the values detected by allozyme markers (F_{ST} = 0.044), probably due to the presence of specific haplotypes in a given population. However, there were at least three haplotypes (h5, h6 and h12) shared by five out of eight of the populations tested at variable frequencies (Table 2), independent of the distance between populations that in some cases exceeded 500 km (Figure 1), reinforcing the hypothesis that gene flow occurred before forest fragmentation, although co-ancestry cannot be ruled out. Auler *et al.* (2002) has reported that *A. angustifolia* allozyme data detected a low level of population structure (F_{st} 0.044 and Gst 0.056) as compared with that revealed by chloroplast markers (G_{ST} 0.28). Powell *et al.* (1995) has shown that in the conifer *Pinus leucodermis* (Ant.) there is a high level of population differentiation in polymorphic simple sequence repeat (SSR) regions from organelles as compared with the SSR of nuclear DNA. These authors also pointed out that, as

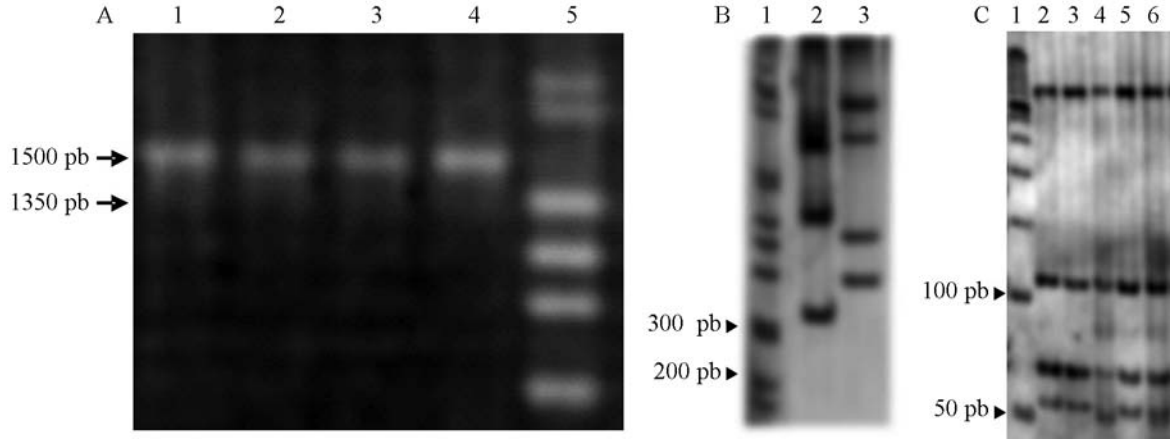

Figure 2 - A: The cpDNA PCR product of the non-coding CS region of megagametophytes and embryos of *Araucaria angustifolia* seeds. Lanes: (1) megagametophyte 1; (2) embryo 1; (3) Megagametophyte 2; (4) embryo 2; and (5) molecular marker λ digested with *Hind*III/*Hae*III. B, C: Restriction fragment patterns of the cpDNA PCR products from *Araucaria angustifolia*. B: (1) 1 kb molecular marker; (2) and (3) patterns of the AS PCR product digested with *RsaI,* from a polymorphic (2) and a non-polymorphic individual (3). C: (1) 100 pb molecular marker; (2 to 6) patterns of the CS PCR products digested with *HinfI,* from non-polymorphic (2, 3 and 5) and polymorphic (4 and 6) individuals.

Table 2 - Number (n) and frequencies *(f)** of the haplotypes found in non-coding regions of the chloroplast DNA in *Araucaria angustifolia*.

Haplotypes (primer/enzyme combination)	Populations†																			
	FAF		RAC		ECA		CUR		FGG		PML		FRA		FTB		Total	
	Haplotype frequency																	
	n	*f*	n	*f*	n	*f*	n	*f*	n	*f*	n	*f*	n	*f*	n	*f*	n	*f*
h1 (AS/RsaI and HinfI)	0	0	0	0	1	0.055		0	0	0	0	0	0	0	0	0	1	0.007
h2 (CS/HinfI, QR/TaqI and RsaI, SfM/HinfI)	1	0.053	0	0	0	0		0	0	0	0	0	0	0	0	0	1	0.006
h3 (CS/HinfI and K1K2/RsaI)	0	0	1	0.056	0	0		0	0	0	0	0	0	0	0	0	1	0.007
h4 (CS/HinfI and RsaI)	0	0	2	0.111	0	0		0	0	0	0	0	4	0.250	0	0	6	0.045
h5 (CS/HinfI)	6	0.316	7	0.389	0	0	12	0.600	0	0	0	0	6	0.375	2	0.100	33	0.222
h6 (CS/RsaI)	1	0.053	4	0.222	0	0	1	0.050	0	0	0	0	3	0.188	3	0.150	12	0.083
h7 (CS/RsaI and SfM/HinfI)	0	0	1	0.056	0	0		0	0	0	0	0	0	0	0	0	1	0.007
h8 (SfM/HinfI)	1	0.05	0	0	0	0		0	0	0	0	0	0	0	0	0	1	0.006
h9 (SfM/TaqI)	0	0	1	0.056	0	0		0	0	0	0	0	0	0	0	0	1	0.007
h10 (SfM/TaqI)	0	0	0	0	0	0		0	1	0.056	0		0	0	0	0	1	0.007
h11 (CS/HaeIII)	0	0	0	0	0	0		0	1	0.056	0	0	0	0	0	0	1	0.007
h12 (monomorphic)	10	0.530	2	0.111	17	0.945	7	0.350	16	0.888	12	1	3	0.187	15	0.750	82	0.595
Total	19	1	18	1	18	1	20	1	18	100	12	1	16		20	1	141	1

*Estimated by the HAPLODIV program (Pons e Petit *et al.*, 1995). †FAF = Fazenda Amola Faca; RAC = Reserva do Caraguatá/Antônio Carlos; ECA = Estação de Caçador; CUR = Curitiba, Paraná; FGG = Fazenda Guamirim Gateado; PML = Parque Municipal de Lages; FRA = Fazenda Rancho Alegre; FTB = Floresta Nacional de Três Barras.

with *A. angustifolia*, *P. leucodermis* used to be widely dispersed but its distribution is now severely restricted, with the high level of population structure revealed in the chloroplast genome being attributed not only to forest degradation but also to low levels of pollen migration between isolated populations and/or genetic drift (Powell *et al.*, 1995). It is interesting to note that cpDNA markers are generally paternally inherited in conifers (Wagner, 1992), therefore the effective population size for the chloroplast genome is half that for nuclear markers and the effects of genetic drift tend to be magnified in organelle markers (Birky, 1989). However, the effects of genetic drift in small populations tend to be countered by long range dispersal of pollen in wind pollinated tree species (Ennos, 1994). In the past there may have been major reductions in *A. angustifolia* population size that allowed genetic drift to reduce genetic diversity and increase genetic differentiation in the remnant populations. Furthermore, although *A. angustifolia* is wind pollinated, its pollen is relatively heavy and therefore, compared to *Pinus* species, pollen dispersal may be relatively limited (Nikles, 1965; Bekessy 2002). If pollen dispersal is restricted in *A. angustifolia* compared to other conifers, it may be more vulnerable to reductions in population size and loss of genetic diversity due to drift. This has implications for the conservation management of this species.

Our results demonstrated that *A. angustifolia* possesses significant within population polymorphism in non-coding regions of its cpDNA, suggesting that the methods used by us could be useful in elucidating the biogeographic patterns of genetic variation, seed and pollen migration of this endangered South American conifer species. However, additional studies with a higher number of populations and trees are essential for a better understanding of gene flow and major trait inheritance in *Araucaria angustifolia*.

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References

Auler NMF, Reis MS, Guerra MP and Nodari RO (2002) The genetics and conservation of *Araucaria angustifolia*: I. Genetic structure and diversity of natural populations by means of non-adaptive variation in the state of Santa Catarina, Brazil. Genet Mol Biol 25:329-338.

Bekessy SA, Allnutt TR, Premoli AC, Lara A, Ennos RA, Burgman MA, Cortes M and Newton AC (2002) Genetic variation in the vulnerable and endemic Monkey puzzle tree, detected using RAPDs. Heredity 88:243-249.

Birky CW Jr, Fuerst P and Maruyama T (1989) Organelle gene diversity under migration, mutation and drift: Equilibrium expectations, approach to equilibrium, effects of hetero-

plasmic cells and comparison to nuclear genes. Genetics 121: 613-627.

Clegg MT (1993) Chloroplast gene sequences and the study of plant evolution. Proc Natl Acad Sci USA 90:363-367.

Demesure B, Sodzi N and Petit RJ (1995) A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Mol Ecol 4:129-131.

Dumolin-Lapègue S, Pemonge M-H and Petit RJ (1997) An enlarged set of consensus primers for the study of organelle DNA in plants. Molecular Ecology 6: 393-7.

Ennos, RA (1994) Estimating the relative rates of pollen and seed migration among plant populations. Heredity 72: 250-259.

Ferreira EF and Grattaplaglia D (1995) Introdução ao Uso de Marcadores mMoleculares RAPD e RFLP em Análise Genética. 2nd edition. EMBRAPA-CENARGEN, Brasília, 220 pp.

Hipkins VD, Krutovskii KV and Strauss SH (1994) Organelle genomes in conifers: Structure, evolution and diversity. Forest Genetics 1:179-189.

Nikles, DG (1965) Progeny tests in slash pine (*Pinus elliottii* Engelm.) in Queensland, Australia. Proc. 8th South. Conf. Forest Tree Improvement, p 112-121.

Pons O and Petit JR (1995) Estimation, Variance and optimal sampling of gene diversity I. Haploid locus. Theor Appl Genet 90:462-470.

Powell W, Morgante M, McDevitt R, Vendramin GG and Rafalski AJ (1995) Polymorphic simple sequence repeat regions in chloroplast genome: Applications to the population genetics of pines. Proc Natl Acad Sci USA 92:7759-7763.

Seitz RA (1986) Crow development of *Araucária angustifolia* in its natural environment during sixty years. Crow and canopy structure in relation to productivity. In: Fugimori T and Whitehead D (Eds) Forestry and Forest Products Research Institute. Ibaraki, Tokyo, p 129-145.

Stefenon VM, Nodari RO and Guerra MP (2004) Genética e conservação de *Araucaria angustifolia*: III. Método rápido de extração de DNA e capacidade informativa de marcadores RAPD para análise da diversidade genética em populações naturais. Biotemas 17:47-63.

Szmidt AE and Wang XR (1994) Molecular systematics and genetic differentiation of *Pinus sylvestris* (L.) and *P. densiflora* (Sieb. Et Zucc.). Theor Appl Genet 86:159-165.

Taberlet P, Gielly L, Pautou G and Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Biol 17:1105-1109.

Wagner DB (1992) Nuclear, chloroplast and mitochondrial DNA polymorphisms as biochemical markers in population genetic analysis of Forest trees. New Forest 6:373-390.

Wakasugi T, Tsudzuki J, Ito S, Nakashima K, Tsudzuki T and Sugiura M (1994) Loss of All *ndh* Genes as Determined by Sequencing the Entire Chloroplast Genome of the Black Pine *Pinus thunbergii*. Proc Natl Acad Sci USA 91:9794-9798.

Wang XR and Szmidt AE (1994) Hybridization and Chloroplast DNA variation in a *Pinus* species complex from Asia. Evolution 48:1020-1031.

Wang XR, Szmidt AE and Lu MZ (1995) Genetic evidence for the presence of cytoplasmic DNA in pollen and megagametophytes and maternal inheritance of mitochondrial DNA in *Pinus.* Forest Genetics 3:37-44.

Wolfe KH, Li WH and Sharp PM (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast and nuclear DNAs. Proc Natl Acad Sci USA 84:9054-9058.

Ziegenhagen B, Schauerte M, Kormuták A and Scholz F (1996) Plastid DNA polymorphism of megagametophytes and pollen in two *Abies* species. Silvae Genet 45:5-6.

Internet Resources

IBAMA (1992) Portaria 37-N, de 03 de abril de 1992. http://www.mma.gov.br/port/sbf/fauna/index.html.

IUCN (2004) International Union for the Conservation of Nature and Natural Resources Red Book. http://www.iucn.org/themes/ssc/publications/red_list/Red_List_2004_book.pdf.

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