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 = 0.612) and an average within-population diversity (hₚ) 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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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 that 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 (Figure 1, Table 1). The total DNA from leaf, megagametophytes and embryos, both isolated from seeds, were extracted using a

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, SM, 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 Hinfl, HindIII, Rsal, HaeIII, TaqI, BamlI, Knpl and EcoRI 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 (hT), within-population diversity (hS), and the differentiation index GST 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 coancestry 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 leucoderms* (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](image-url)
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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