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PRIMER NOTE

# Isolation and characterization of microsatellite markers for *Araucaria angustifolia* (Araucariaceae)

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## Abstract

***Araucaria angustifolia* is a dioecious tree species that occurs in the southern part of Brazil. Because of the intense exploitation of the species due to its valuable wood, only 2% of the original population still remains. Twenty-nine species-specific and highly polymorphic microsatellite loci were developed from a genomic library enriched for AG/TC repeats. Levels of polymorphism were evaluated using a total of 16 adult trees from a natural population. An average of 8.1 alleles per locus was detected, and expected heterozygosity ranged from 0.63 to 0.72.**

*Keywords*: *Araucaria angustifolia*, conservation, genetic diversity, microsatellite

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*Araucaria angustifolia* (Bert.) O. Kuntze is a native dioecious species of the Tropical Atlantic Forest. Natural populations or plantations occur mainly in the three southernmost states of Brazil (Paraná, Santa Catarina and Rio Grande do Sul) where it is called 'pinheiro-do-Paraná', 'araucaria' or 'pinheiro brasileiro'. This species has also spread throughout other states such as São Paulo and Minas Gerais and in Argentina, particularly in the Province of Missiones (Klein 1960).

Due to its relevant ecological, economic and social functions, it is considered one of the most important trees in its region of natural occurrence. Populations of adult individuals create a microcosmic environment where shade-tolerant plant species of other taxa can grow and develop. Their seeds feed the wild fauna, including birds and rodents, which are the main araucaria seed dispersers. Humans also use them for food, in addition to a source of income (Auler *et al*. 2002). Its high quality wood, which gives it its main economic value, can be used for almost everything, especially housing, furniture and pulp (Reitz *et al*. 1978). However, most of the remaining araucarias, about 1–3% (Guerra *et al*. 2002), are still under pressure of exploitation pressure by the timber industry.

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Microsatellite loci or simple sequence repeats (SSR) exhibit high levels of variability because of differences in the number of repeated units. The high allelic diversity and abundance of microsatellites in the eukaryotic genome make these codominant molecular markers popular for detailed genetic studies as genetic diversity and genetic structure (Chase *et al*. 1996).

Total genomic DNA was extracted from leaves of a single individual of *Araucaria angustifolia.* Microsatellite markers were developed from an enriched genomic library for poly $(TC)_{13}$ constructed with *Mse*I-digested DNA. Fragments were separated on a 2% agarose gel, and those from 200 to 800 bp were eluted, ligated to adaptors and used to construct an enriched genomic library, according to protocols previously described (Brondani *et al*. 1998; Collevatti *et al*. 1999). Selected fragments were ligated into pGEM-T Easy vector (Promega) and vectors were used to transform competent *Escherichia coli* XL1-Blue cells, and which were cultivated on 1× Luria-Bertani (LB) agar containing ampicillin, Xgal and IPTG. A total of 400 colonies were screened as positive for (TC)n repeats and amplified with M13 forward primer. Plasmid DNA was sequenced using dye-terminator fluorescent chemistry and products detected on an ABI PRISM 377 automated sequencer (Applied Biosystems).

**Table 1** Primer sequences of the microsatellite marker loci of *Araucaria angustifolia* are listed with annealing temperatures ($T_a$), number of the alleles per locus ($A$), expected heterozygosity ($H_E$), observed heterozygosity ($H_O$) and respective GenBank Accession numbers

| Locus | Array | Primer sequence (5′–3′) | Allele size range (bp) | $T_a$ (°C) | $N$ | $A$ | $H_E$ | $H_O$ | GenBank Accession nos |
|---|---|---|---|---|---|---|---|---|---|
| Aang01 | $(CT)_{22}$ | F: 5′TGACGGGTTCACTCCTACCT3′<br>R: 5′TAGGAACCCCCATTCATTTG3′ | 200–260 | 56 | 16 | 8 | 0.81 | 0.87 | AY865575 |
| Aang02 | $(GA)_{27}$ | F: 5′AAGGGCCAGAATGAAAAGGT3′<br>R: 5′TTCACCCCCACATATTGTTGT3′ | 250–290 | 56 | 16 | 7 | 0.63 | 0.44 | AY865576 |
| Aang03 | $(CT)_{13}$ | F: 5′CGCCTACCTCAATCACTGGT3′<br>R: 5′TGGGACAATGTGCTTATCCA3′ | 150–170 | 56 | 16 | 7 | 0.62 | 0.37 | AY865577 |
| Aang04 | $(GA)_{12}$ | F: 5′TTGAAACCAACCATGATCCA3′<br>R: 5′GTTTTCCATTGCGATGTGG3′ | 150–170 | 56 | 15 | 4 | 0.25 | 0.27 | AY865578 |
| Aang07 | $(GA)_{24}$ | F: 5′ACCTCACAGGGACACCTCAC3′<br>R: 5′TTTTCATGCATGTTGCTTGC3′ | 200–280 | 54 | 15 | 11 | 0.88 | 0.80 | AY865579 |
| Aang09* | $(GA)_{12}$ | F: 5′TCTTCTACAATAGCTCATTCCCTT<br>R: 5′TGAGGAGAGGGAAGAGAAGGT3′ | 150–170 | 54 | 12 | 6 | 0.79 | 0.25 | AY865580 |
| Aang12* | $(GA)_{23}$ | F: 5′AAGGGTTCACAATGCTGAGG3′<br>R: 5′TGGATTTTATTATGATGGTTGTTCC3′ | 190–240 | 56 | 9 | 9 | 0.87 | 0.55 | AY865581 |
| Aang13 | $(GA)_{20}$ | F: 5′AAGGGTTCACAATGCTGAGG3′<br>R: 5′TGGATTTTATTATGATGGTTGTTCC3′ | 200–230 | 56 | 16 | 5 | 0.73 | 0.44 | AY865582 |
| Aang14 | $(GA)_{27}$ | F: 5′GAGCACGTGCAGATGTTGAT3′<br>R: 5′CCATCCTCTCCATGACCACT3′ | 150–190 | 56 | 13 | 11 | 0.81 | 0.61 | AY865583 |
| Aang15 | $(GA)_{19}$ | F: 5′TGGTCGATCGTAGGGATCAT3′<br>R: 5′GCTGTCGAGCCTCCTATCAC3′ | 210–290 | 56 | 16 | 13 | 0.91 | 0.94 | AY865584 |
| Aang17* | $(GA)_{22}$ | F: 5′TAAAAAGGGTGCAAATGTGG3′<br>R: 5′TTGTCATGGTCGCATCTTGT3′ | 250–290 | 56 | 16 | 9 | 0.87 | 0.50 | AY865585 |
| Aang18* | $(TC)_9$ | F: 5′ACACGTTTAATCAGACGAAGAAG3′<br>R: 5′ATGCCACCTTTTTTCAGCAAC3′ | 190–320 | 54 | 12 | 12 | 0.93 | 0.50 | AY865586 |
| Aang21 | $(CT)_{12}$ | F: 5′GGAGACACCTCACCCCCTA3′<br>R: 5′TGATGAGGGAGGATTACAAGC3′ | 190–210 | 56 | 15 | 7 | 0.80 | 0.67 | AY865587 |
| Aang22 | $(GA)_{10}$ | F: 5′TCAACTTGCAAGGTCACCTCTA3′<br>R: 5′ATGGGAGCCCCTTCTAGTGT3′ | 220–250 | 56 | 14 | 6 | 0.53 | 0.43 | AY865588 |
| Aang23 | $(GA)_{19}$ | F: 5′TGAGGTATTTGGGCTAGCAA3′<br>R: 5′CTTCCACGCTCTCACTTTCC3′ | 180–200 | 56 | 15 | 5 | 0.46 | 0.47 | AY865589 |
| Aang24 | $(CT)_{19}$ | F: 5′CTCTCCTTCCCCTTGCTCTT3′<br>R: 5′AGGTGGATCACCCACTGAAG3′ | 160–200 | 56 | 14 | 8 | 0.86 | 0.64 | AY865590 |
| Aang27 | $(CT)_{12}$ | F: 5′CATGGTGGCTATTGCTCCTT3′<br>R: 5′AGAAGCCATCAAAGGAGTGG3′ | 160–210 | 56 | 16 | 11 | 0.87 | 0.81 | AY865591 |
| Aang28 | $(CT)_{11}$ | F: 5′TCCATTGCATTAGTTTGGGATA3′<br>R: 5′TTTCCAATCATACATTCACCACA3′ | 130–170 | 58 | 12 | 10 | 0.91 | 0.92 | AY865592 |
| Aang30 | $(CT)_{21}$ | F: 5′GTGGAGGTCTTGGCTAATGG3′<br>R: 5′TAGCTGGGAGCTGATCCAAT3′ | 210–230 | 56 | 9 | 5 | 0.71 | 0.78 | AY865593 |
| Aang35* | $(GA)_{10}$ | F: 5′GGTGAAGCTTCGTTTCAAGG3′<br>R: 5′CCACTTGTCTTCACCAACCA3′ | 200–270 | 56 | 14 | 11 | 0.89 | 0.93 | AY865594 |
| Aang36 | $(GA)_{14}$ | F: 5′CACCCCCTGTAGGATTCAAA3′<br>R: 5′ATGGTTGCTGATGATGACGA3′ | 175–215 | 56 | 15 | 9 | 0.81 | 0.67 | AY865595 |
| Aang37 | $(GA)_{18}$ | F: 5′GGGGAGTTTCCATGAGATGA3′<br>R: 5′TCCACTCACCACTCTGAGGA3′ | 250–270 | 54 | 15 | 4 | 0.25 | 0.20 | AY865596 |
| Aang41 | $(GA)_{12}$ | F: 5′TTGTCCATGTGAACGAGTCC3′<br>R: 5′TCTCTCCATTATTCATACATGCTC3′ | 170–300 | 56 | 16 | 14 | 0.91 | 0.94 | AY865597 |
| Aang42 | $(GA)_{15}$ | F: 5′TGCACCAATGAACACCACTT3′<br>R: 5′GCCCACCAACTACCACCATA3′ | 140–160 | 56 | 16 | 6 | 0.77 | 0.94 | AY865598 |
| Aang43 | $(GA)_{24}$ | F: 5′AGGCTCACATCAGGCTCACT3′<br>R: 5′TGGTTTTGGTGGTCAAATCA3′ | 160–190 | 56 | 16 | 8 | 0.53 | 0.44 | AY865599 |
| Aang44* | $(CT)_{15}$ | F: 5′CAGAGGGTGGACACTTGGTT3′<br>R: 5′CACAAACCCCTTTTGCCTAA3′ | 250–280 | 54 | 16 | 6 | 0.73 | 0.19 | AY865600 |
| Aang45 | $(CT)_{15}$ | F: 5′AGGCTCACATCAGGCTCACT3′<br>R: 5′TGGTTTTGGTGGTCAAATCA3′ | 190–270 | 54 | 16 | 11 | 0.83 | 1.00 | AY865601 |
| Aang46 | $(CT)_{12}$ | F: 5′TCCACCTACCTCAATCACTGG3′<br>R: 5′TGGGACAATGTGCTTATCCA3′ | 210–230 | 56 | 16 | 5 | 0.69 | 0.81 | AY865602 |
| Aang47 | $(GA)_{15}$ | F: 5′GATATGAAAAGAAGGGTTCTATGCT3′<br>R: 5′TTTCTTCCATTCCTCCAAGC3′ | 155–175 | 58 | 16 | 6 | 0.72 | 0.87 | AY865603 |

*Hardy–Weinberg disequilibrium expectations ($P < 0.05$).

The results indicated that 399 colonies had a microsatellite with adequate size and position in the cloned inserts. From these 399 positive clones, 80 (20%) contained both microsatellite and appropriate flanking regions for primer design of unique sequence. Primer complementary to flanking microsatellite sequences were designed using the PRIMER3 Output software (Rozen & Skaletsky 2000). Fifty pairs of them were synthetized, optimized and utilized to estimate the number of alleles ($A$), observed heterozygosity ($H_O$) and expected heterozygosity ($H_E$) with 16 adult trees from five natural populations of *Araucaria angustifolia*.

Microsatellite loci were amplified using polymerase chain reaction (PCR) in 13-μL volumes containing 3 ng of genomic DNA, 0.25 mM of each dNTPs, 1.5 mM $MgCl_2$, 1× PCR buffer (10 mM Tris-HCL, 50 mM KCl), 0.25 mg/mL BSA, 0.25 μM of each primer, and 1 U of *Taq* DNA polymerase (Gibco). Amplifications were performed using an MJ Research PTC-100 thermal controller using the following protocol: denaturation at 94 °C for 5 min; 29 cycles of denaturation at 94 °C for 1 min, annealing temperature ($T_a$) (Table 1) for 1 min, and extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. Reaction products were separated on polyacrylamide gel and visualized by silver staining. Alleles were sized relative to a 10-bp ladder. Number of alleles per locus, mean expected and observed heterozygosities were calculated using Genetic Data Analysis version 1.0 (GDA) (Lewis & Zaykin 2001).

We successfully amplified products with 29 (58%) primer pairs, out of 50. All microsatellite marker loci were highly polymorphic revealing on average 8.1 alleles per locus, and expected and observed heterozygosity mean values of 0.72 and 0.63, respectively. Six loci (Aang09, Aang12, Aang17, Aang18, Aang35 and Aang44) showed departure from Hardy–Weinberg expectations after correcting for multiple tests (Bonferroni method, $P < 0.0017$). Moreover, significant linkage disequilibrium was detected for four (Aang02/Aang03, Aang04/Aang09, Aang04/Aang43 and Aang03/Aang44) out of the 406 pairwise comparisons. It should be noted, however, that deviations from equilibrium might be due to our limited sampling.

The high levels of polymorphism make the present primers useful for population genetic studies. We are currently using these markers to investigate questions of levels of genetic differentiation between natural populations and genetic diversity, as part of studies to Units of Conservation created in the southern of Brazil.

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