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Investigating the Mendelian inheritance, genetic linkage, and genotypic disequilibrium for ten microsatellite loci of *Araucaria angustifolia*

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Abstract

Araucaria angustifolia is a dioecious and wind pollinated conifer that typically occurs in higher attitudes of Southern Brazil. After a significant reduction of its population during the twentieth century, public policies have enabled natural populations to recover. As new studies focus on the genetics of the species it is important to investigate Mendelian inheritance, genetic linkage, and genotypic disequilibrium for the microsatellite loci developed for the species. Here we analyze ten microsatellite loci developed for *A. angustifolia* by genotyping 295 adult trees and 13 open pollinated progenies from a forest fragment in Santa Catarina, Brazil. The likelihood G-test shows a perfect 1:1 Mendelian segregation for all ten loci, indicating that these molecular markers are genetic markers. Significant genetic linkage between pairwise loci was detected in only 3% of the tests, suggesting that these loci are not located in the same linkage groups within the chromosomes. However, genotypic disequilibrium was detected in 51% of pairwise loci for adult trees, probably due to the strong spatial genetic structure of the population. Our results indicate that the ten loci analyzed can be used in studies on genetic diversity and structure, mating system, and gene flow of the species.

Key words: Araucaria; Conservation genetics; Microsatellites; pinheiro-do-paraná; Tropical tree species.

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Introduction

With its unique crown shape and wide dispersion across Southern Brazil, the conifer *Araucaria angustifolia* is typical of the region's landscape. Its valuable timber was a focus of intensive logging during the twentieth century causing a dramatic reduction in its natural population. The species is currently classified as threatened with extinction on the Brazilian Red List (MARTINELLI and MORAES, 2013) and logging of the species is forbidden. Its protection has led to a gradual recovery in which new, natural populations are currently found across the region which has increased interest in the conservation of the species. Current methodologies, especially those using microsatellite markers (SSR), have been conducted for many tree species due to their high degree of polymorphism in terms of number of alleles (ASHLEY, 2010), which allow for a number of genetic analyses. Several current studies have examined existing populations of *A. angustifolia* for genetic variability, mating, and pollen flow; however, in order to validate the results obtained from the use of such molecular markers, it is essential to determine if the SSRs developed for the species have their loci linked and if their inheritance follows the Mendelian segregation assumptions.

Araucaria angustifolia (Bert.) O. Kuntze (Araucariaceae) – Brazilian pine – is a wind pollinated conifer, with seeds dispersed mainly by barochory (but with some zoochory by agoutis, birds, and squirrels). The species is mainly dioecious and it typically occurs at altitudes between 500 to 2300 m above sea level (asl). Because of its dominant position in the canopy, it is characteristic of the forest type, commonly known as Araucaria Forest (OMBROPHYLOUS MIXED FOREST; IBGE, 2012). The species presents pioneer-like behavior in abandoned areas and grasslands but it can also act as a

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Table 1. – Mendelian inheritance tests for ten microsatellite loci in progenies of *Araucaria angustifolia*.

Locus	Seed-tree	Genotype	n_1	$n_{ij} : n_{ii} + n_j$	G_1	n_2	$n_{ik} : n_{jk}$	G_2	Locus	Seed-tree	Genotype	n_1	$n_{ij} : n_{ii} + n_j$	G_1	n_2	$n_{ik} : n_{jk}$	G_2
CRCA									Ag56								
C1b	11	97101	7	3:4	NE	21	3:18	11.89		11	145151	8	7:1	NE	20	11:9	0.20
	12	9399	9	9:0	NE	19	5:14	4.44		12	145147	0	0:0	NE	28	9:19	3.65
	14	9399	11	7:4	0.83	17	7:10	0.53		13	139145	4	2:2	NE	24	6:18	6.28
	15	9399	15	12:3	5.78	13	5:8	0.70		14	145147	7	7:0	NE	21	11:10	0.05
	16	9399	15	11:4	3.40	13	4:9	1.97		15	141147	4	4:0	NE	24	6:18	6.28
	20	9399	11	6:5	0.09	17	6:11	1.49		16	141147	5	5:0	NE	23	9:14	1.10
	21	9397	24	17:7	4.30	4	3:1	NE		20	147149	9	9:0	NE	19	4:15	6.78
	22	9397	14	8:6	0.29	14	0:14	NE		21	145149	11	9:2	4.82	18	10:8	0.22
	24	9399	17	10:7	0.53	11	2:9	4.82		22	141147	3	3:0	NE	25	9:16	1.99
	25	9597	13	2:11	6.86	15	11:4	3.40		24	141147	2	2:0	NE	26	10:16	1.40
	26	9399	12	4:8	1.36	16	10:6	1.01		25	147149	6	6:0	NE	22	12:10	0.18
	27	9397	22	14:8	1.66	6	5:1	NE		26	147149	10	9:1	7.36	18	4:14	5.88
	31	9397	22	15:7	2.98	6	3:3	NE		27	145149	7	6:1	NE	21	10:11	0.05
	32	9397	28	20:8	5.31	0	0:0	NE		31	149151	1	0:1	NE	27	19:8	4.61
	33	9397	20	16:4	7.71	8	5:3	NE		32	147149	12	12:0	NE	16	3:13	6.74
	36	9397	28	23:5	12.54	0	0:0	NE		33	147149	11	10:1	8.55	17	8:9	0.06
	37	9397	28	22:6	9.72	0	0:0	NE		36	147149	12	10:2	5.82	16	3:13	6.74
	38	8991	0	0:0	NE	28	11:17	1.30		37	147149	13	11:2	6.86	15	3:12	5.78
	39	9397	27	19:8	4.61	0	0:0	NE		39	147149	13	13:0	NE	15	1:14	13.45*
Ag45	11	165169	16	10:6	1.01	12	6:6	0.0	AS90	11	175181	14	9:5	1.16	15	10:5	1.70
	12	157165	6	5:1	NE	22	3:19	12.97		12	173177	9	9:0	NE	19	8:11	0.48
	13	159165	25	22:3	16.31*	3	2:1	NE		13	173177	15	13:2	9.01	13	1:12	10.97
	14	159165	17	11:6	1.49	11	4:7	0.83		14	175177	15	10:5	1.70	13	6:7	0.08
	15	159165	12	9:3	3.14	16	7:9	0.25		15	173177	12	12:0	NE	16	5:11	2.31
	16	159165	15	11:4	3.40	14	5:9	1.16		16	173177	14	13:1	12.20	14	4:10	2.66
	20	159165	14	7:7	0.0	14	3:11	4.86		20	175177	17	12:5	2.97	11	6:5	0.09
	21	159165	22	6:16	4.72	6	0:6	NE		21	171175	7	5:2	NE	21	5:16	6.06
	22	159165	11	8:3	2.36	17	9:8	0.06		22	175177	15	11:4	3.40	13	6:7	0.08
	24	159165	9	8:1	NE	19	9:10	0.05		24	173177	8	8:0	NE	20	8:12	0.81
	25	163165	28	20:8	5.31	0	0:0	NE		26	175177	12	8:4	1.36	16	6:10	1.01
	26	159165	14	6:8	0.29	14	4:10	2.66		27	175177	10	8:2	3.85	18	8:10	0.22
	27	159165	19	4:15	6.78	9	3:6	NE		31	171175	5	3:2	NE	22	7:15	2.98
	31	159165	20	6:14	3.29	8	2:6	NE		32	175177	23	17:6	5.48	5	4:1	NE
	32	163165	26	18:8	3.95	2	0:2	NE		33	175177	17	15:2	11.25	11	6:5	0.09
	33	163165	28	13:15	0.14	0	0:0	NE		36	175177	23	20:3	14.07*	5	2:3	NE
	38	163165	28	11:17	1.30	0	0:0	NE		37	175177	22	18:4	9.64	6	3:3	NE
	39	163165	26	14:12	0.15	2	0:2	NE		38	175177	12	12:0	NE	16	9:7	0.25
										39	171175	6	6:0	NE	22	7:15	2.98
Locus	Seed-tree	Genotype	n_1	$n_{ij} : n_{ii} + n_j$	G_1	n_2	$n_{ik} : n_{jk}$	G_2	Locus	Seed-tree	Genotype	n_1	$n_{ij} : n_{ii} + n_j$	G_1	n_2	$n_{ik} : n_{jk}$	G_2
CRCAC																	
1a	11	193201	11	4:7	0.83	17	5:12	2.97	AG20	11	241245	3	3:0	NE	25	13:12	0.04
	12	195201	14	6:8	0.29	14	2:12	7.92		12	235243	9	5:4	NE	19	6:13	2.64
	13	195201	22	10:12	0.18	0	0:0	NE		13	239243	7	6:1	NE	21	9:12	0.43
	14	195201	21	7:14	2.38	0	0:0	NE		14	233243	14	6:8	0.29	14	1:13	12.20
	15	195201	22	6:16	4.72	0	0:0	NE		15	243247	9	6:3	NE	19	14:5	4.44
	16	195201	20	8:12	0.81	0	0:0	NE		16	243247	14	3:11	4.86	14	13:1	12.20
	21	193201	15	6:9	0.60	0	0:0	NE		20	243249	11	8:3	2.36	17	9:8	0.06
	22	195201	11	5:6	0.09	0	0:0	NE		21	235243	24	6:18	6.28	4	1:3	NE
	24	195201	16	4:12	4.19	0	0:0	NE		22	235243	13	6:7	0.08	15	5:10	1.70
	25	199201	27	9:18	3.06	0	0:0	NE		24	243247	17	12:5	2.97	11	9:2	4.82
	27	195201	13	2:11	6.86	0	0:0	NE		25	243245	19	11:8	0.48	9	5:4	NE
	31	195201	16	9:7	0.25	0	0:0	NE		26	243249	14	7:7	0.0	14	10:4	2.66
	32	201203	22	16:6	4.72	6	5:1	NE		27	243249	8	2:6	NE	20	4:16	7.71
	33	201203	17	11:6	1.49	11	9:2	4.82		31	233243	15	8:7	0.07	13	4:9	1.97
	36	199201	27	9:18	3.06	1	0:1	NE		32	243249	27	10:17	1.84	0	0:0	NE
	37	201203	17	15:2	11.25	11	7:4	0.83		33	243245	22	14:8	1.66	6	6:0	NE
	38	201203	21	11:10	0.05	7	7:0	NE		36	243245	22	17:5	6.92	5	3:2	NE
	39	199201	28	16:12	0.57	0	0:0	NE		37	243249	24	11:13	0.17	4	4:0	NE
										38	243249	28	7:21	7.33	0	0:0	NE
Aang28																	
a	11	150154	12	8:4	1.36	16	7:9	0.25	Aang28b	11	164168	24	17:7	4.30	4	4:0	NE
	12	152154	14	6:8	0.29	14	7:7	0.0		12	164168	27	16:11	0.93	1	0:1	NE
	13	148152	20	13:7	1.83	8	3:5	NE		13	166168	11	11:0	NE	17	1:16	15.96*
	14	154156	6	2:4	NE	23	12:11	0.04		14	166168	12	12:0	NE	16	8:8	0.0
	15	150152	21	12:9	0.43	7	4:3	NE		15	166170	13	6:7	0.08	15	15:0	NE
	16	148150	6	5:2	NE	22	15:7	2.98		16	164168	27	19:8	4.61	1	1:0	NE
	20	152154	18	11:7	0.90	11	7:4	0.83		20	164170	5	5:0	NE	23	12:11	0.04
	22	150152	14	7:7	0.0	13	7:6	0.08		21	166168	10	9:1	7.36	19	6:13	2.64
	24	150156	12	5:7	0.33	16	8:8	0.0		22	166168	17	14:3	7.72	11	3:8	2.36
	25	148154	23	12:11	0.04	5	1:4	NE		24	164168	24	11:13	0.17	4	2:2	NE
	26	150152	17	12:5	2.97	11	7:4	0.83		25	164168	20	12:8	0.81	8	5:3	NE
	27	150152	9	9:0	NE	19	10:9	0.05		26	164168	18	9:9	0.0	10	10:0	NE
	31	150152	13	10:3	3.98	15	8:7	0.07		27	164170	18	11:7	0.90	10	10:0	NE
	32	148152	16	13:3	6.74	12	5:7	0.33		31	166168	12	12:0	NE	16	4:12	4.19
	33	150152	13	9:4	1.97	15	8:7	0.07		32	164168	25	14:11	0.36	3	2:1	NE
	36	148152	17	9:8	0.06	11	2:9	4.82		36	166170	10	10:0	NE	18	11:7	0.90
	37	148152	14	13:1	12.20	14	9:5	1.16		37	166168	14	13:1	12.20	14	4:10	2.66
	38	148152	16	16:0	NE	12	6:6	0.0		38	164168	27	17:10	1.84	1	1:0	NE
										39	164168	26	17:9	2.50	2	2:0	NE

n_1 and n_2 = sample size; G_1 and G_2 = maximum likelihood G statistics for the hypothesis of $n_{ij} = n_{ii} + n_{jj}$ and $n_{ik} : n_{jk}$, respectively.
* Significance after Bonferroni correction for $\alpha = 0.05$ ($\chi 2 = 13.33$). NE = not estimated for n_1 and n_2 was lower than 10.

partially shade tolerant tree in forest environments. Because of its long life cycle, natural regeneration might be uncommon for long periods until gaps are formed in the forest canopy allowing for sapling development. On the other hand, the regeneration of the species is believed to be compromised by several factors such as dominance of invasive species in the understory (e.g., native bamboos), seed and seedling predation, and seed

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Continuation of *Table 1.*

Locus	Seed-tree	Genotype	n_1	$n_{ii} : n_{ii} - n_i$	G_1	n_2	$n_{ik} : n_{jk}$	G_2	Locus	Seed-tree	Genotype	n_1	$n_{ij} : n_{ii} + n_j$	G_1	n_2	$n_{ik} : n_{ik}$	G_2
Aang01	11	212218	3	3:0	NE	25	11:14	0.36	Aang14	11	205211	19	8:11	0.48	9	0:9	NE
	12	216222	13	9:4	1.97	15	3:12	5.78		12	211217	21	11:10	0.05	6	2:4	NE
	13	218230	2	1:1	NE	26	9:17	2.50		13	209211	22	8:14	1.66	6	0:6	NE
	14	212216	11	9:2	4.82	17	9:8	0.06		14	211213	19	12:7	1.33	9	4:5	NE
	15	208216	12	8:4	1.36	16	9:7	0.25		15	207213	20	6:14	3.29	8	0:8	NE
	16	210216	9	5:4	NE	19	8:11	0.48		20	205219	0	0:0	NE	28	12:16	0.57
	20	206212	21	9:12	0.43	7	0:7	NE		21	209211	12	8:4	1.36	16	9:7	0.25
	22	214216	15	8:7	0.07	13	7:6	0.08		24	211215	12	8:4	1.36	16	5:11	2.31
	24	212222	7	4:3	NE	21	9:12	0.43		26	211213	24	17:7	4.30	4	1:3	NE
	25	208222	13	6:7	0.08	15	13:2	9.01		27	213217	6	5:1	NE	22	14:8	1.66
	26	214216	4	3:1	NE	24	11:13	0.17		31	209211	9	6:3	NE	19	8:11	0.48
	27	214216	2	2:0	NE	26	10:16	1.40		32	211215	15	8:7	0.07	13	5:8	0.70
	32	212216	6	3:3	NE	22	14:8	1.66		33	211217	18	11:7	0.90	10	5:5	0.0
	33	208212	11	5:6	0.09	17	8:9	0.06		36	209217	5	3:2	NE	23	22:1	23.66*
	36	200216	2	2:0	NE	26	13:13	0.0		37	211217	15	9:6	0.60	13	9:4	1.97
	37	208226	12	8:4	1.36	16	11:5	2.31		38	211217	18	7:11	0.90	10	6:4	0.40
	38	216226	3	3:0	NE	25	15:10	1.01		39	215217	6	4:2	NE	22	11:11	0.0
	39	208214	16	9:7	0.25	12	2:10	5.82									

n_1 and n_2 = sample size; G_1 and G_2 = maximum likelihood G statistics for the hypothesis of $n_{ij} = n_{ii} + n_{ij}$ and $n_{ik} : n_{jk}$, respectively. * Significance after Bonferroni correction for $\alpha = 0.05$ ($\chi 2 = 13.33$). NE = not estimated for n_1 and n_2 was lower than 10.

collection for human consumption (e.g., LACERDA et al., 2012).

The reduction of continuous habitats into small forest fragments and problems with regeneration can cause an immediate decrease in genetic diversity due to the loss of alleles (WHITE et al., 1999). This is associated with a reduction in population size (CASCANTE et al., 2002), disruptions to natural mating systems, and interruptions to gene flow. In turn, these processes are linked to an increase in inbreeding levels and population divergence (JUMP and PENUELAS, 2006; BITTENCOURT and SEBBENN, 2007; SEBBENN et al., 2011). In this context, the reduction of continuous *A. angustifolia* habitats into small forest fragments may interrupt gene flow and cause an immediate decrease in genetic diversity (BITTENCOURT and SEBBENN, 2007; 2009). Thus, the genetic conservation of the species has become a priority that must be supported by studies of genetic diversity and structure as well as gene flow. Despite efforts to address areas of genetics analyses, such as pollen flow, mating systems, and effects of fragmentation on genetic variability, it is essential to confirm that basic assumptions regarding Mendelian inheritance, genetic linkage, and genotypic disequilibrium are met. As such, we aim to assess the Mendelian inheritance, genetic linkage, and genotypic disequilibrium for ten microsatellite loci developed for *A. angustifolia*.

Materials and Methods

The study was carried out in a 7 ha dense cluster of *A. angustifolia* inside the Embrapa Research Station in Caçador (ERSC), Santa Catarina State, Brazil. All 295 *A. angustifolia* trees in the study area had their vascular cambium sampled. We also sampled open-pollinated seeds from 13 randomly selected seed trees in the cluster and eight trees located in the adjacent open forest. From each seed tree, we genotyped 28 seeds from a single cone, with the exception of one tree located in the open forest, from which only 14 seeds were genotyped.

The isolation of DNA from *A. angustifolia* seeds followed the protocol described by MAZZA and BITTENCOURT (2000). The same protocol was applied to the vascular cambium tissue from adult trees with minor modifications (removal of proteinase K and CTAB 10% (NaCl 1.4 M) solutions). Quantification was performed comparing 5 µL of the DNA from each sample with 5 µL of Phage Lambda DNA size marker with concentrations of 20, 50 and 100 ng in 2% agarose gel. After quantification, each sample was diluted with autoclaved Milli-Q water to a final concentration of 10 ng/µL to begin tests with SSR markers. Of the 20 pairs of microsatellite markers available for *A. angustifolia*, eight were selected to be amplified in three multiplex systems.

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

The method described by GILLET and HATTEMER (1989) was used to investigate the Mendelian inheritance of the *A. angustifolia* SSR loci, which is based on comparisons of a heterozygous maternal genotype tree with the segregation of its alleles in open-pollinated progeny.

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Table 2. – Maximum likelihood G-test for the hypothesis of independent segregation between pairwise loci (1:1:1:1) in progenies of *Araucaria angustifolia*. ST is the seed tree.

ST	CRCAC1b								AG56							
	Ang56	AG45	AS90	CRCAC 1a	AG20	Aang28 a	Aang28 b	Aang01	Aang14	AG45	AS90	CRCA C1a	AG20	Aang28 a	Aang28b	Aang01	Aang14
11	7.79	4.10	10.07	9.90	2.58	1.46	5.28	15.81	23.26	1.63	5.12	6.63	2.86	3.61	1.42	6.54	13.31
12	7.66	13.33	2.80	16.43	9.73	1.33	4.47	10.30	9.91	6.70	2.08	0.88	4.91	2.14	1.75	5.69	3.43
13	NE	NE	0.67	4.81	NE	0.73	4.59	NE	NE	9.93	8.09	8.84	11.42	17.48	7.29	1.51	20.56
14	2.07	10.09	0.13	26.53*	22.36	2.44	1.37	0.20	0.78	1.82	0.38	7.10	17.92	2.07	3.80	0.90	3.92
15	4.65	5.00	1.13	8.90	4.04	0.72	11.00	1.69	16.17	13.37	6.96	12.31	6.07	8.99	11.91	8.64	NE
16	7.31	16.46	0.59	13.31	18.20	3.25	0.38	9.52	NE	3.15	2.10	9.02	12.96	5.56	2.42	1.82	NE
20	1.18	12.19	4.71	NE	7.66	2.99	13.92	9.47	5.53	6.00	3.78	NE	8.26	3.22	2.57	2.95	3.89
21	3.54	16.50	4.54	7.22	21.69	NE	5.35	NE	0.47	9.21	6.29	6.49	16.78	NE	6.52	NE	1.77
22	5.42	11.14	8.96	26.19	16.61	9.23	10.37	10.96	NE	1.62	5.24	15.45	3.02	3.88	2.92	4.49	NE
24	12.30	7.18	1.85	11.02	2.42	7.48	0.90	0.06	5.01	6.94	4.09	11.09	2.36	15.39	8.55	4.63	3.36
25	18.82	8.29	10.90	15.67	24.52*	23.60*	1.87	10.34	NE	2.20	1.92	1.90	1.81	13.73	1.51	12.67	NE
26	9.57	7.57	1.52	NE	7.00	1.14	2.15	2.77	1.18	10.06	5.00	NE	8.21	2.40	3.90	0.24	0.50
27	5.96	20.83	0.32	10.40	4.87	0.29	7.00	5.00	10.48	11.55	0.05	7.99	2.00	0.63	16.90	0.78	7.24
31	5.42	8.29	5.12	4.69	9.96	1.70	1.69	NE	2.02	6.78	1.62	5.65	5.86	4.11	6.74	NE	1.36
32	4.93	2.10	3.10	1.88	7.98	3.29	9.03	7.39	3.35	1.20	2.39	2.51	17.24	6.03	1.59	9.95	3.12
33	2.98	8.02	0.38	4.96	19.45	0.66	6.21	2.91	2.36	0.86	1.17	3.62	31.74*	1.67	0.13	1.81	0.90
36	6.28	3.17	0.76	16.94	0.95	7.57	8.50	0.72	26.31*	0.90	1.67	3.91	1.21	9.87	0.44	2.81	22.48
37	2.49	6.98	1.92	3.39	20.34	3.87	2.81	6.55	5.62	1.75	1.81	1.63	19.46	1.21	0.03	8.90	2.84
38	1.88	18.59	11.81	3.47	3.36	6.12	6.14	4.18	1.48	1.61	1.11	11.27	20.20	3.08	2.09	2.35	2.33
39	2.91	4.64	7.58	6.89	NE	NE	3.14	3.72	1.20	7.67	3.36	10.64	NE	NE	1.83	2.57	0.96

ST	AG45							AS90					
	AS90	CRCA C1a	AG20	Aang28 a	Aang28b	Aang01	Aang14	CRCAC 1a	AG20	Aang28 a	Aang28b	Aang01	Aang14
11	1.74	9.05	4.21	2.87	6.18	1.97	8.95	8.27	1.12	2.69	1.17	13.57	19.63
12	12.95	9.92	10.22	2.37	9.88	4.53	19.22	6.61	7.14	6.44	6.78	4.63	5.65
13	5.55	5.64	0.81	2.10	10.71	1.52	21.85	8.64	9.02	10.99	7.85	2.97	18.97
14	2.95	5.64	19.04	0.50	2.21	1.35	0.55	12.30	18.01	2.70	5.88	2.02	2.66
15	2.33	6.32	6.82	1.31	10.80	0.30	11.12	7.69	8.38	1.33	10.69	2.33	11.65
16	2.18	6.35	16.14	3.01	1.22	3.02	NE	7.77	12.18	3.95	1.59	0.51	NE
20	11.31	NE	8.91	7.58	13.54	14.05	11.90	NE	3.02	4.15	2.91	8.09	0.83
21	18.06	27.13*	30.25*	NE	22.77	NE	17.37	11.04	23.74*	NE	15.27	NE	9.51
22	2.61	15.69	4.51	0.96	1.58	1.82	NE	7.95	3.61	3.92	0.70	4.11	NE
24	0.58	4.45	4.12	6.03	3.66	0.38	1.75	3.00	15.50	0.47	7.59	0.68	4.20
25	1.46	11.99	4.27	7.13	4.53	9.71	NE	16.76	3.75	6.60	4.37	7.98	NE
26	10.18	NE	12.08	10.98	10.32	3.13	9.71	NE	4.15	2.52	3.03	4.16	3.55
27	8.86	25.04*	10.92	25.35*	16.15	8.15	15.46	9.95	1.56	0.91	8.68	2.99	2.15
31	22.92	18.37	22.78	13.62	14.78	NE	17.61	6.07	12.04	4.95	8.82	NE	13.20
32	4.92	3.22	18.68	2.80	6.86	5.05	1.53	10.65	17.65	1.73	3.03	4.79	1.77
33	3.65	7.96	31.36*	5.00	1.48	3.69	7.27	4.63	16.02	1.21	0.04	0.46	1.23
36	4.15	14.18	4.92	4.05	1.47	3.25	29.41	15.95	1.11	8.46	3.79	0.65	22.55
37	2.06	0.83	27.53*	3.82	0.65	7.27	8.40	2.88	NE	0.50	0.53	6.63	7.30
38	1.43	11.00	30.09*	1.48	0.92	2.15	3.46	9.13	5.83	0.31	1.38	2.46	0.52
39	6.87	15.15	NE	NE	5.01	5.55	2.80	8.55	NE	NE	8.07	8.80	1.36

ST	CRCAC1a					AG20				Aang28a			Aang28b		Aang01 Aang14
	AG20	Aang28 a	Aang28b	Aang01	Aang14	Aang28 a	Aang28b	Aang01	Aang14	Aang28a	Aang01	Aang14	Aang01	Aang14	
11	7.65	13.00	14.22	4.09	NE	5.97	7.31	2.40	11.63	10.81	1.77	23.63*	0.55	24.02	11.50
12	22.43	4.63	9.61	18.07	14.75	3.77	7.69	6.70	9.17	5.17	4.97	3.80	10.17	11.84	9.34
13	3.95	14.47	10.03	1.30	18.78	0.27	5.28	0.88	16.89	7.12	3.46	16.53	5.09	1.92	8.66
14	26.00*	1.55	8.01	2.16	5.01	17.37	26.56*	14.80	16.30	2.91	1.60	4.75	1.25	0.28	4.57
15	8.98	11.61	11.02	7.53	20.06	6.48	17.10	3.72	8.70	16.91	0.21	23.47*	6.55	13.72	11.31
16	11.59	10.03	7.85	3.87	NE	4.25	20.98	8.65	28.91*	4.25	3.20	NE	1.35	NE	NE
20	NE	NE	NE	NE	NE	5.82	7.80	8.75	10.75	5.00	9.93	1.18	14.42	3.71	17.44
21	25.27*	NE	13.85	NE	11.31	NE	20.14	8.75	NE	NE	NE	NE	NE	1.28	NE
22	19.48	6.68	6.03	8.57	NE	4.60	3.38	3.17	6.30	1.64	3.03	NE	0.58	NE	NE
24	5.59	7.35	11.30	2.28	5.64	9.41	8.61	3.52	NE	2.82	3.88	2.49	1.75	3.75	1.12
25	6.62	13.22	14.56	16.23	NE	10.38	1.41	6.85	4.86	4.45	NE	NE	11.79	NE	NE
26	NE	NE	NE	NE	NE	6.03	3.86	8.22	9.46	0.83	2.43	4.07	0.53	5.94	5.93
27	21.85	7.89	18.27	2.30	11.37	7.80	11.04	0.90	11.38	3.65	0.36	1.21	5.70	18.17	4.60
31	10.07	1.26	2.37	NE	2.47	6.78	10.97	NE	8.38	0.38	NE	5.17	NE	2.40	NE
32	17.31	1.37	4.89	4.96	0.52	7.59	8.65	8.53	21.41	9.74	11.59	2.14	4.82	3.22	7.59
33	33.67*	5.01	4.77	6.38	7.22	17.65	28.24*	20.99	34.37*	0.29	0.97	2.80	6.83	5.05	2.35
36	9.37	28.63*	2.19	12.04	NE	12.34	2.31	2.52	17.69	12.99	1.73	14.16	1.53	20.19	5.71
37	28.52*	0.11	1.89	5.84	5.16	10.42	28.12*	15.82	18.89	7.25	9.92	1.19	7.36	4.87	8.19
38	NE	5.61	6.15	10.11	6.78	18.15	12.62	10.09	NE	2.29	2.95	2.65	3.02	1.40	4.80
39	NE	NE	15.63	8.55	2.03	NE	NE	NE	NE	NE	NE	NE	3.15	0.50	4.43

* Significance after Bonferroni correction for $\alpha = 0.05$. 0.00029 ($\chi 2 = 23.28$). G = G-test for three degrees of freedom. NE = not estimated.

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This method assumes that the loci have regular segregation and its alleles follow classic Mendelian inheritance patterns based on three main requirements: 1) regular meiotic segregation during ovule production; 2) random ovule fertilization by type of pollen; 3) no selection occurring between the moment of fertilization and genotyping of the seeds. The model also assumes that there is a co-dominant relationship among all alleles. The method further requires that the following conditions are met: 1) all progeny of a tree must possess a maternal allele; and 2) in cases of heterozygous parent trees (e.g. A_iA_j, i ≠ j): a) each offspring must possess an allele of the maternal tree, A_i or A_j; b) the number of heterozygous progeny A_iA_j (n_{ij}) must be equal to the sum of homozygous progeny A_iA_i (n_{ii}) and A_jA_j (n_{jj}), or $n_{ij} = n_{ii} + n_{jj}$; and c) the number of heterozygous progeny A_iA_k (n_{ik}) must equal the number of heterozygous progeny A_jA_k (n_{ik}), or $n_{ik} = n_{jk}$, where k ≠ i, j. Based on this model, and using the open-pollinated progenies sampled from 25 seed-trees during two reproductive events, we compared the segregation observed in each progeny of the heterozygous maternal tree for a given loci with the expected hypothesis of regular Mendelian 1:1 segregation, the G-test (SOKAL and ROHLF, 1981), based on the following formula (Equation 1):

$$G = 2\left[n_{ij} \ln\left(\frac{n_{ij}}{E(n)}\right) + (n_{ii} + n_{ij}) \ln\left(\frac{(n_{ii} + n_{jj})}{E(n)}\right)\right] \quad \text{(Equation 1)}$$

where ln is the natural logarithm, and $E(n)$ is the expected number of genotypes for the alleles A_iA_j (n_{ij}) and $A_iA_i + A_jA_j$ ($n_{ii} + n_{jj}$), based on: $E(n) = 0.5(n_{ij} + n_{ii} + n_{jj})$ or based on Equation 2:

$$G = 2\left[n_{ik} \ln\left(\frac{n_{ik}}{E(n)}\right) + n_{jk} \ln\left(\frac{n_{jk}}{E(n)}\right)\right] \quad \text{(Equation 2)}$$

where, $E(n)$ is the expected number of genotypes for the alleles A_iA_k (n_{ik}) and A_jA_k (n_{jk}), based on: $E(n) = 0.5(n_{ik} + n_{jk})$. Additionally, a Bonferroni correction for multiple comparisons (95%, α = 0.05) was used to avoid false positives.

To test whether the loci of progeny were genetically linked, a linkage test was carried out between pairwise loci, using genetic information from parent trees that were doubly-heterozygous for two loci. In this case, the null hypothesis (H_0) is the regular Mendelian 1:1:1:1 segregation. The hypothesis of regular segregation between pairwise loci was accepted or discarded based on a maximum likelihood G-test (SOKAL and ROHLF, 1981), shown in Equation 3, performed for each progeny:

$$G = 2\left[n_{ik} \ln\left(\frac{n_{ik}}{E(n)}\right) + n_{il} \ln\left(\frac{n_{il}}{E(n)}\right) + n_{jk} \ln\left(\frac{n_{jk}}{E(n)}\right) + n_{jl} \ln\left(\frac{n_{jl}}{E(n)}\right)\right] \quad \text{(Equation 3)}$$

where, n_{ik}, n_{il}, n_{jk}, and n_{il} are the observed number of phenotypes A_iB_k, A_iB_l, A_jB_k, and A_jB_l, respectively; $E(n)$ is the expected number of genotypes A_iB_k, A_iB_l, A_jB_k, and A_jB_l, respectively; is the natural logarithm; and $E(n)$ is calculated as $E(n) = 0.25(n_{ik} + n_{il} + n_{jk} + n_{il})$. The Bonferroni correction for multiple comparisons (95%, α = 0.05) was also applied.

The genotypic disequilibrium test was carried out for adult trees, since genotypic disequilibrium is expected in progeny arrays because descendants always receive a maternal allele. The genotypic disequilibrium test was carried out using the FSTAT program (GOUDET, 2002) associated with a Bonferroni correction (95%, α = 0.05).

Results

After the Bonferroni correction, significant deviations from the expected 1:1 Mendelian segregation pattern were detected in only five (2%) of 251 tests (*Table 1*). After the Bonferroni correction, 25 of the 807 linkage tests (3%) were significant (*Table 2*), suggesting absence of linkage between pairwise loci. However, the genotypic

Table 3. – Results for the genotypic disequilibrium analysis between pairwise microsatellite loci from adult trees of *Araucaria angustifolia.*

Pairwise loci	Adult trees
CRCAC1bXAG56	0.00016
CRCAC1bXAG45	0.00016
CRCAC1bXAS90	0.00016
CRCAC1bXCRCAC1a	0.00016
CRCAC1bXAG20	0.00016
CRCAC1bXAang28a	0.00016
CRCAC1bXAang28b	0.04429
CRCAC1bXAang01	0.00016
CRCAC1bXAang14	0.00159
AG56XAG45	0.00016
AG56XAS90	0.00016
AG56XCRCAC1a	0.00016
AG56XAG20	0.00016
AG56XAang28a	0.02254
AG56XAang28b	0.67206
AG56XAang01	0.00302
AG56XAang14	0.15635
AG45XAS90	0.00016
AG45XCRCAC1a	0.00016
AG45XAG20	0.00016
AG45XAang28a	0.03079
AG45XAang28b	0.01016
AG45XAang01	0.05794
AG45XAang14	0.00810
AS90XCRCAC1a	0.00016
AS90XAG20	0.00016
AS90XAang28a	0.01413
AS90XAang28b	0.21429
AS90XAang01	0.00381
AS90XAang14	0.07460
CRCAC1aXAG20	0.00016
CRCAC1aXAang28a	0.36746
CRCAC1aXAang28b	0.55587
CRCAC1aXAang01	0.14746
CRCAC1aXAang14	0.07381
AG20XAang28a	0.04492
AG20XAang28b	0.00016
AG20XAang01	0.00016
AG20XAang14	0.02492
Aang28aXAang28b	0.00016
Aang28aXAang01	0.00016
Aang28aXAang14	0.04460
Aang28bXAang01	0.00016
Aang28bXAang14	0.02889
Aang01XAang14	0.00016

The values represent the probability of genotypic disequilibrium after 1200 permutations of alleles among individuals. Probability after Bonferroni correction: P = 0.00016; α = 0.05.

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disequilibrium was detected in 51% of pairwise loci for adult trees (*Table 3*), probably due to the presence of strong spatial genetic structure of the population.

Discussion

The results showed that the ten microsatellite loci segregated according to the Mendelian rules of 1:1. In general, the five cases where deviations were detected occurred at different loci in the same progenies, suggesting that the observed deviations can be attributed to sample drift, considering that *A. angustifolia* cones contain many seeds (> 200) and the maximum sample size was 28 seeds per tree. Furthermore, the deviation occurred in only one of the realized tests ($n_{ij} : n_{ii} + n_{jj}$ or $n_{ik} : n_{jk}$), indicating that these deviations are random and these loci segregate according to Mendelian rules. Thus, the ten microsatellite markers can be considered genetic markers. Similarly, Mendelian segregation for the loci Ag45, AS90, CRCAC1a, Ag20, Aang28a, Aang01 and Aang14 was previously validated by Danner et al. (2013).

Linkage between some pairs of loci was detected for a limited number of tests. The significant linkage between pairwise loci for some progenies came from individual locus deviations from the 1:1 Mendelian segregation because progenies with Mendelian deviations in some loci were used for the linkage tests. For example, the progeny of seed tree 36 showed deviations from the 1:1 Mendelian segregation for locus Aang14 and linkage with CRCAC1b, indicating that the segregation deviations in an individual locus may have generated the significant G-test values. Thus, all loci can be used for population genetic analyses such as genetic diversity and structure, mating system, and gene flow.

Evidence of genotypic disequilibrium between pairs of loci in adult trees was detected in 51% of the tests, suggesting high linkage disequilibrium. The genotypic disequilibrium is typical of a population and is caused by many factors including small sample size, selfing, mating among relatives, correlated mating, bottlenecks and founder effects, artificial and natural selection, and intrapopulation spatial genetic structure. Because the size of our sample population is high (295) and the tree species is dioecious and long-living (no selfing occurs), we believe that intrapopulation spatial genetic structure was the cause of the observed genotypic disequilibrium between many pairs of loci.

As a conclusion, the ten loci assessed herein present Mendelian inheritance and are not linked. Thus, these loci can be used in population genetic analyses, especially in mating system and parentage analysis studies.

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