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Mendelian segregation in eight microsatellite loci from hand- and open-pollinated progenies of *Araucaria angustifolia* (Bert.) O. Kuntze (Araucariaceae)

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Abstract

In order to use molecular markers in population genetics studies, it is important to confirm that the molecular markers used present a Mendelian segregation. The aim of this paper was to investigate the genetic segregation of eight microsatellite loci of *Araucaria angustifolia* (Bert.) O. Kuntze (Araucariaceae). The study was carried out comparing genetic segregation in hand- and open-pollinated progenies of maternal dioecious and monoecious trees. The Mendelian segregation was confirmed for all eight loci studied (Ag20, Ag23, Ag45, Aang01, Aang14, Aang28, As90 and CRCAc1), as no deviation from the expected segregation hypothesis was detected in the studied progenies. Therefore, these eight loci can be used for further population genetics studies of *A. angustifolia*.

Key words: Brazilian pine, artificial pollination, open pollination, monoecy.

Introduction

Araucaria (*Araucaria angustifolia* (Bert.) O. Kuntze) (Araucariaceae) is a dioecious, wind pollinated conifer that is rarely monoecious. The species is endemic to the South and Southeast of Brazil, with small fragments of the species occurring in Argentina and Paraguay (HUECK, 1972). The species is currently considered endangered (MMA, 2008) since its population area in Brazil has been reduced to less than 3% of the 18.2 million hectares recorded in the late nineteenth century (HUECK, 1972; GUERRA et al., 2002). The species is ecologically important because adult trees create shady conditions under the canopy which are necessary for the regeneration of other native species (BARBOSA et al., 2009). Moreover, the Araucaria seeds are a crucial source of food for animals that inhabit Araucaria forests

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as the seeds provide an excellent source of energy, particularly in winter (VIDOLIN et al., 2011). Humans also consume the seeds and the harvesting and selling of seeds is a significant source of annual income for hundreds of low-income families in Southern Brazil (SILVA and REIS, 2009).

Knowledge of the mating system, gene flow, genetic structure and diversity of a species are fundamental in developing strategies for the conservation and breeding of tree species (SEBBENN, 2006). Such information can be efficiently assessed based on data from molecular markers, such as SSR (simple sequence repeat), also known as microsatellites. However, for molecular markers to be used in population genetics studies, it is important to confirm that the loci have Mendelian segregation in order to avoid bias in the estimates of genetic parameters (HATTEMER and GILLET, 1989).

Segregation is how a pair of alleles is separated during meiosis in gametes and how alleles are distributed in a given population. To check for deviations in segregation, loci from hand-pollinated progenies of known parental genotypes (full-sib progeny) and open-pollinated progenies from heterozygous maternal genotypes (progeny which may contain mixtures full-sibs, half-sibs, and selfing) can be compared.

The objective of this study was to investigate the segregation of eight microsatellite loci of *A. angustifolia* in order to determine their suitability for future studies of the mating system, gene flow, genetic structure and diversity of the species. The study was conducted comparing the segregation of hand- and open-pollinated progenies from dioecious and monoecious maternal trees.

Material and Methods

Sampling

To obtain the *A. angustifolia* progenies, hybridizations were performed between 2005 and 2006, following the methods described in ANSELMINI and ZANETTE (2012). Hand-pollinations were developed (*Table 1*), using five sampled female individuals (PF_1, PF_3, PF_Social, PF_Solos and PF_Fazenda, all located in the city of Curitiba, Paraná) and six male individuals as pollen donors (Gua_1 and Gua_2, located in the Parque das Araucárias in Guarapuava, Paraná; PB_1 located in Pato Branco, Paraná; and Lg_Galpão, Lg_Horta and Lg_5, from the Experimental Farm Epagri in Lages, Santa Catarina). Hand-pollination was also performed by inducing selfing of the monoecious individual PF_3 (PF_3 x PF_3). Open-pollinated progenies (with no control over male pollen donors) were obtained from three female dioecious trees (Pinha_400, located in Caçador, Santa Catarina; and PF_Social and PF_Fazenda, located in Curitiba, Paraná) and three monoecious trees (MN_Ara from Aratiba, Rio Grande do Sul; MN_SD, São Domingos, Santa Catarina; and MN_Gua, Guarapuava, Paraná). The mature Araucaria cones resulting from both hand- and open-pollination were harvested and the seeds sown in polythene bags containing soil as substrate. The seedlings of each progeny were labeled separately and were grown outdoors in the Agricultural Sciences complex at the Universidade Federal do Paraná (UFPR), in Curitiba, Paraná.

Needles were collected from the sampled parents as well as 18 offspring (2–3 years old) resulting from the each of the hand-and open-pollination, totaling 321 samples. After collection, the needles were placed in individual plastic bags containing silica gel to remove moisture

Table 1. – Method of pollination, identification of and the spatial distance between *Araucaria angustifolia* parents sampled for the analysis.

Progeny	Female parent	Male parent	Spatial distance between parents (km)
Hand-pollination	PF_3	PB_1	348.3
Hand-pollination	PF_3	Gua_1	223.3
Hand-pollination	PF_3	Lg_Galpão	284.2
Hand-pollination	PF_Social	PB_1	350.1
Hand-pollination	PF_Solos	PB_1	348.4
Hand-pollination	PF_Solos	Lg_Horta	284.5
Hand-pollination	PF_Fazenda	Lg_5	291.8
Hand-pollination	PF_Fazenda	Gua_2	235.3
Hand-pollination	PF_1	Lg_Horta	284.4
Hand-pollination	PF_3	Lg_Horta	284.3
Hand-pollination	PF_3	PF_3	-
Open-pollination	Pinha_400	-	-
Open-pollination	PF_Fazenda	-	-
Open-pollination	PF_Social	-	-
Open-pollination	MN_Ara	-	-
Open-pollination	MN_SD	-	-
Open-pollination	MN_Gua	-	-

PF_3, MN_Ara, MN_SD and MN_Gua are monoecious *A. angustifolia* trees.

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and prevent degradation of DNA. Samples were shipped to the Laboratory of Genetics of Microorganisms (LabGeM), UFPR, and were subsequently lyophilized for 72 hours and stored at –40 °C until DNA isolation.

Microsatellite analysis

DNA isolation from the *A. angustifolia* needles was undertaken using the protocol described in FERREIRA and GRATTAPAGLIA (1996, p.127), with minor modifications. Quantification was performed with DNA from each sample using the NanoDrop spectrophotometer® 2000 (Thermo Scientific). After quantification, each sample was diluted with autoclaved milli-Q water to a final concentration of 10 ng/uL. This study used eight microsatellite loci developed for the Araucaria species which were chosen based on a reported absence of linkage disequilibrium in the original analyses (SCOTT et al., 2003; SALGUEIRO et al., 2005; SCHMIDT et al., 2007). However, no published study exists that describes the inheritance of these loci. To verify the annealing temperature (Ta° C) most suitable for each of the eight loci, we tested 10 DNA samples for each loci using PCR (Polymerase Chain Reaction) and temperature gradients in the Maxygene® thermocycler (Axygen). The PCR product was visualized by electrophoresis on 1.5% agarose gel, applying 5 µL of the PCR product and 2 µL of the GelRed® (Biotium) dye. Electrophoresis was performed with a current of 3 volts/cm for 120 min and the result was visualized using transillumination with ultraviolet light.

For PCR amplification of all samples we used a reaction solution with a final volume of 10 µL, containing 5 µL of Qiagen Multiplex PCR Master Mix 2X, 1 µL of 10X primer mix (2 mM each primer), 2 µL genomic DNA (10 ng l-1), 1 µL of 5X Q-Solution, and 1 µL of Milli-Q water. The PCR program was used in the thermocycler with an initial step of denaturation of DNA and Taq DNA polymerase activation at 95 °C for 15 min, followed by 35 cycles of amplification in three stages (94° C for 30 sec), annealing temperature (*Table 2*) for 90 sec, and 72 °C for 60 seconds), and a final extension at 72 °C for 10 minutes. After amplification we added 10 µL of Milli-Q water to each sample, which were subsequently kept refrigerated at 4 °C until genotyping.

For genotyping of each sample, we used a solution of 10 µL containing 1 µL of the amplified fragment solution, 0.125 µL of GS 500 ROX® standard or 0.5 µL of GS 600 LIZ®, and the remaining volume was made up with formamide Hi-Di®. Polymorphism was detected by labeling primers and PCR biplex or triplex combinations with fluorescent dyes (*Table 2*), followed by detection of the fragments by capillary electrophoresis in 3500xL ABI automated sequencer Genetic Analyzer (Applied Biosystems). The size of the fragments (alleles) were determined by interpretation of the electropherogram peaks generated using the GeneMapper v.4.1 software (Applied Biosystems). The values referring to the sizes of the alleles were exported to spreadsheets for statistical analysis.

Data analysis

To study the segregation of microsatellite loci in *A. angustifolia*, we adopted the method described by GILLET and HATTEMER (1989). We compared the maternal and paternal genotypes (in cases of controlled parentage) with the segregation of the hand- and open-pollinated progeny. The test requires that the following conditions must be met. Firstly, all progeny of a tree A_iA_i must possess the A_i allele from the mother tree. Secondly, in cases of heterozygous parent trees (e.g. A_iA_i, i≠j): a) among offspring, each individual must possess an allele of the maternal tree, A_i and A_j; b) the number of heterozygous progeny $A_iA_j(n_{ij})$ must equal 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 $A_jA_k(n_{jk})$, or $n_{ik}=n_{jk}$, where $k\neq$i, j. The phenotypes observed in each heterozygous offspring were compared with the expected segregation by event of 1:1 or 1:2:1, by means of a maximum likelihood G-test using the following formula:

Table 2. – Primers labeled with fluorescence without combination or in biplex or triplex combination for genetic analysis of *A. angustifolia*.

Combination	Fluorescence	Detected fragment (pb)	Annealing temperature
Ag20	FAM (blue)	240-258	59.3° C
Triplex			
Ag45	FAM (blue)	154-168	57.8° C
CRCAc1	FAM (blue)	203-225	57.8° C
As90	NED (yellow)	160-180	57.8° C
Biplex			
Aang01	HEX (green)	200-260	56° C
Aang14	FAM (blue)	150-190	56° C
Biplex			
Aang28	HEX (green)	130-170	55° C
Ag23	FAM (blue)	245-259	55° C

500 ROX® GS was used to size samples with the loci Ag20, Ag45, As90 and CRCAc1 and GS 600 LIZ® for samples with loci Ag23, Aang01, Aang14 and Aang28.

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$$G = 2\left[n_i \ln\left(\frac{n_i}{E(n)}\right) + n_j \ln\left(\frac{n_j}{E(n)}\right)\right] \quad \text{(Weir, 1996)},$$

where, n_i and n_j are the observed number of genotypes containing alleles A_i and A_j, respectively, $E(n)$ is the expected number of genotypes for the allele A_i and A_j, where $E(n) = 0.5\ (n_i + n_j)$, ln is the natural logarithm. The *G*-test determines if the deviation between the observed and expected segregation is statistically significant or if deviations may be explained by chance. We also applied the Bonferroni correction for multiple comparisons (95%, $\alpha = 0.05$) to avoid false positives. The frequency of alleles at all loci were calculated and classified into three categories: (a) high frequency ($p > 0.25$); (b) intermediate frequency ($0.05 < p \leq 0.25$); and (c) rare frequency ($p \leq 0.05$). The alleles unique to each progeny type were also identified

Results and Discussion

Of the 321 genotyped samples, only seven (2.2%) did not demonstrate sufficient quality for unambiguous genotype detection. This result does not significantly interfere with the segregation analysis.

Among open-pollinated progenies, all loci had private alleles except for CRCAc1. Among hand-pollinated progenies, only two private alleles were found: one allele in the Aang14 locus and one in the Aang28. All loci also showed one or two alleles with high frequency ($p > 0.25$),

Table 3. – Allele frequencies and private alleles of eight microsatellite loci in hand-pollinated progenies ($n = 192$) and open-pollinated progenies ($n = 107$) of *Araucaria angustifolia*.

Locus	Allele	Hand-pollination	Open-pollination	Locus	Allele	Hand-pollination	Open-pollination
Ag20	239	0.05	0.07	Ag23	247	0.07	0.09
	241	0.08	0.06		249	0.55	0.44
	243	0.71	0.67		251	0.04	0.06
	245	0.00	0.01		253	0.07	0.07
	249	0.16	0.16		255	0.27	0.28
	253	0.00	0.03		257	0.00	0.06
	Private	0	2		Private	0	1
Ag45	151	0.00	0.04	CRCAc1	199	0.10	0.14
	163	0.19	0.17		201	0.79	0.74
	165	0.81	0.79		203	0.11	0.12
	Private	0	1		Private	0	0
Aang01	200	0.00	0.02	Aang14	148	0.00	0.02
	202	0.00	0.07		150	0.02	0.04
	204	0.00	0.02		152	0.09	0.15
	206	0.00	0.02		154	0.00	0.01
	208	0.32	0.07		156	0.00	0.01
	210	0.02	0.01		160	0.00	0.01
	212	0.04	0.10		162	0.04	0.07
	214	0.00	0.20		164	0.29	0.26
	216	0.17	0.10		168	0.15	0.05
	218	0.23	0.11		170	0.26	0.25
	220	0.02	0.05		172	0.00	0.08
	222	0.06	0.04		174	0.06	0.02
	224	0.11	0.09		176	0.00	0.01
	226	0.02	0.01		180	0.00	0.01
	230	0.00	0.07		182	0.02	0.02
	232	0.00	0.01		184	0.06	0.00
	244	0.00	0.01		188	0.00	0.01
	Private	0	8		Private	1	8
Aang28	134	.00	0.02	As90	173	0.05	0.08
	136	0.06	0.00		175	0.31	0.32
	144	0.00	0.01		177	0.44	0.22
	150	0.13	0.18		179	0.06	0.09
	152	0.12	0.22		181	0.14	0.22
	154	0.21	0.07		183	0.00	0.06
	156	0.02	0.03		189	0.00	0.01
	158	0.04	0.02		-	-	-
	160	0.00	0.01		-	-	-
	162	0.07	0.09		-	-	-
	166	0.26	0.12		-	-	-
	168	0.10	0.23		-	-	-
	Private	1	3		Private	0	2

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except for the open-pollinated progenies with locus Aang01, for which there were eight alleles of intermediate frequency ($0.05 < p \leq 0.25$) and nine rare alleles ($p > 0.05$). In some loci, no rare alleles were found, including the hand-pollinated progenies with loci Ag45 and CRCAc1, and open-pollinated progenies with loci Ag23 and CRCAc1 *(Table 3)*. The loss of low frequency alleles in fragmented *A. angustifolia* populations at a rate higher than continuous, well preserved forest (e.g. Mangueirinha, Paraná) was reported in BITTENCOURT and SEBBENN (2009). Therefore, the presence of private and low frequency alleles detected in the current study demonstrates the potential of open-pollinated progenies for *ex situ* genetic conservation and breeding programs. However, it should be noted that these progenies are the maternal sibs of only six trees (three female and three monoecious), located in three different Brazilian states. Thus, the differentiation of alleles is expected due to the effects of long distances (WRIGHT, 1943). Although the microsatellite markers used in this study are neutral,

Table 4. – Mendelian inheritance in eight microsatellite loci in hand-pollinated progenies of *Araucaria angustifolia*.

Locus/Hybrid	Genotype	n	$n_{ij}:n_{ii}+n_{ij}$	$n_{ik}:n_{jk}$	$n_{il}:n_{ijl}$	G	Df	Locus/Hybrid	Genotype	n	$n_{ij}:n_{ii}+n_{ij}$	$n_{ik}:n_{jk}$	$n_{il}:n_{ijl}$	G	df
Ag20								Ag45							
PF 3xPB 1	243/249x243/249	18	10:8	0:0	0:0	2.32	2	PF 3xPB 1	163/165x165/165	18	7:22	0:0	0:0	0.90	1
PF 3xGua 1	243/249x239/243	17	7:6	2:2	0:0	5.09	3	PF 3xGua 1	163/165x163/165	17	4:13	0:0	0:0	5.72	2
PF_3xLg_Galpão	243/249x239/243	18	5:5	5:3	0:0	0.73	3	PF_3xLg_Galpão	163/165x163/165	18	4:14	0:0	0:0	5.66	2
PF_SocialxPB_1	239/241x 243/249	17	0:0	6:5	2:4	2.26	3	PF_SocialxPB_1	163/165x165/165	17	7:10	0:0	0:0	0.53	1
PF_SolosxPB_1	241/243x243/249	17	4:3	0:0	4:6	1.08	3	PF_SolosxPB_1	165/165x165/165	17	0:17	0:0	0:0	-	-
PF SolosxLg Horta	241/243x243/243	16	5:11	0:0	0:0	2.31	1	PF SolosxLg Horta	165/165x165/165	16	0:16	0:0	0:0	-	-
PF_FazendaxLg_5	243/243x241/243	18	0:10	8:0	0:0	0.22	1	PF_FazendaxLg_5	165/165x163/165	18	0:10	8:0	0:0	0.22	1
PF FazendaxGua 2	243/243x243/243	17	0:17	0:0	0:0	-	-	PF FazendaxGua 2	165/165x165/165	17	0:17	0:0	0:0	-	-
PF 1xLg Horta	243/243x243/243	18	0:18	0:0	0:0	-	-	PF 1xLg Horta	165/165x165/165	18	0:18	0:0	0:0	-	-
PF_3xLg_Horta	243/249x243/243	18	612:	0:0	0:0	2.04	1	PF_3xLg_Horta	163/165x165/165	18	7:11	0:0	0:0	0.90	1
PF 3xPF 3	243/249x243/249	18	6:12	0:0	0:0	2.04	2	PF 3xPF 3	163/165x163/165	18	9:9	0:0	0:0	0.00	2
Ag23								Aang01							
PF_3xPB_1	249/255x249/253	18	5:4	0:0	5:4	0.22	3	PF_3xPB_1	208/218x208/216	18	6:6	0:0	3:3	2.04	3
PF 3xGua 1	249/255x249/249	17	7:10	0:0	0:0	0.53	1	PF 3xGua 1	208/218x208/222	17	5:4	0:0	4:4	0.17	3
PF 3xLg Galpão	249/255x249/251	18	4:5	0:0	4:5	0.22	3	PF 3xLg Galpão	208/218x212/212	18	0:0	10:8	0:0	0.22	1
PF_SocialxPB_1	249/255x249/253	17	5:4	0:0	4:4	0.17	3	PF_SocialxPB_1	216/220x208/216	17	5:4	4:4	0:0	0.17	3
PF_SolosxPB_1	247/255x249/253	17	0:5	5:4	5:3	0.68	3	PF_SolosxPB_1	218/222x208/216	17	0:0	5:4	4:4	0.17	3
PF_SolosxLg_Horta	247/255x249/249	16	0:0	8:8	0:0	0.00	1	PF_SolosxLg_Horta	218/222x216/224	16	0:0	4:4	4:4	0.00	3
PF FazendaxLg 5	249/255x247/249	18	5:4	5:4	0:0	0.22	3	PF FazendaxLg 5	208/224x208/210	18	5:0	0:0	4:5	0.22	3
PF_FazendaxGua_2	249/255x249/251	17	5:4	0:0	4:4	0.17	3	PF_FazendaxGua_2	208/224x218/218	17	0:0	9:8	0:0	0.06	1
PF_1xLg_Horta	249/255x249/249	18	10:8	0:0	0:0	0.22	1	PF_1xLg_Horta	216/226x216/224	18	4:5	0:0	4:5	0.22	3
PF_3xLg_Horta	249/255x249/249	18	10:8	0:0	0:0	0.22	1	PF 3xLg Horta	208/218x216/224	18	0:0	4:6	4:4	0.63	3
PF 3xPF 3	249/255x249/255	18	8:4	0:0	0:0	0.22	2	PF 3xPF 3	208/218x208/218	18	5:13	0:0	0:0	3.68	2
Aang14								As90							
PF 3xPB 1	164/170x168/184	18	0:0	5:5	4:4	0.22	3	PF 3xPB 1	175/177x177/177	18	12:6	0:0	0:0	2.04	1
PF 3xGua 1	164/170x152/164	17	4:5	4:4	0:0	0.17	3	PF 3xGua 1	175/177x175/181	17	3:3	0:0	6:5	1.58	3
PF_3xLg_Galpão	164/170x164/182	18	7:2	0:0	4:5	3.05	3	PF_3xLg_Galpão	175/177x177/177	18	8:10	0:0	0:0	0.22	1
PF_SocialxPB_1	164/170x168/184	17	0:0	4:6	3:4	1.08	3	PF_SocialxPB_1	173/175x177/177	17	0:0	8:9	0:0	0.06	1
PF_SolosxPB_1	152/174x168/184	17	0:0	5:3	6:3	1.58	3	PF_SolosxPB_1	179/181x177/177	17	0:0	9:0	0:0	0.06	1
PF SolosxLg Horta	152/174x164/168	16	0:0	5:4	3:4	0.51	3	PF SolosxLg Horta	179/181x175/175	16	0:0	7:9	0:0	0.25	1
PF FazendaxLg 5	162/170x164/170	18	3:9	4:6	0:0	1.13	3	PF FazendaxLg 5	177/181x177/181	18	7:11	0:0	0:0	0.99	2
PF_FazendaxGua_2	162/170x150/170	17	4:9	5:4	0:0	0.17	3	PF_FazendaxGua_2	177/181x177/179	17	4:4	0:0	4:5	0.17	3
PF 1xLg Horta	152/174x164/168	18	0:0	5:5	3:5	0.73	3	PF 1xLg Horta	173/177x175/175	18	0:0	10:8	0:0	0.22	1
PF 3xLg Horta	164/170x164/168	18	4:5	0:0	4:5	0.22	3	PF 3xLg Horta	175/177x175/175	18	11:7	0:0	0:0	0.90	1
PF_3xPF_3	164/170x164/170	18	8:5	0:0	0:0	0.22	2	PF_3xPF_3	175/177x175/177	18	7:11	0:0	0:0	0.90	2
Aang28								CRCAc1							1
PF 3xPB 1	154/166x136/168	18	0:0	4:4	5:5	0.22	3	PF 3xPB 1	201/201x199/201	18	0:9	9:0	0:0	0.00	-
PF_3xGua_1	154/166x156/162	17	0:0	5:4	4:4	0.17	3	PF_3xGua_1	201/201x201/201	17	0:17	0:0	0:0	-	1
PF_3xLg_Galpão	154/166x152/168	18	0:0	4:5	5:4	0.22	3	PF_3xLg_Galpão	201/201x201/203	18	0:11	0:0	7:0	0.90	2
PF_SocialxPB_1	150/152x136/168	17	0:0	4:4	4:5	0.17	3	PF_SocialxPB_1	199/201x199/201	17	7:10	0:0	0:0	0.93	1
PF SolosxPB 1	152/158x136/168	17	0:0	4:4	5:4	0.17	3	PF SolosxPB 1	201/201x199/201	17	0:9	8:0	0:0	0.06	1
PF SolosxLg Horta	152/158x150/166	16	0:0	4:4	4:4	0.00	3	PF SolosxLg Horta	201/201x201/203	16	0:9	0:0	7:0	0.25	1
PF_FazendaxLg_5	154/162x150/166	18	0:0	5:4	5:4	0.22	3	PF_FazendaxLg_5	201/201x201/203	18	0:7	0:0	11:0	0.90	-
PF FazendaxGua 2	154/162x150/152	17	0:0	4:4	4:5	0.17	3	PF FazendaxGua 2	201/201x201/201	17	0:17	0:0	0:0	-	3
PF 1xLg Horta	154/166x150/166	18	5:4	4:5	0:0	0.22	3	PF 1xLg Horta	199/201x201/203	18	4:5	0:0	4:5	0.22	1
PF_3xLg_Horta	154/166x150/166	18	4:5	4:5	0:0	0.22	3	PF_3xLg_Horta	201/201x201/203	18	0:11	0:0	7:0	0.90	-
PF 3xPF 3	154/166x154/166	18	9:9	0:0	0:0	0.00	2	PF 3xPF 3	201/201x201/201	18	0:18	0:0	0:0	-	

n is the sample size. G is the value of the maximum likelihood G-test for the hypotheses $n_{if}=n_{ii}+n_{ij}$ and $n_{ik}=n_{jk}$. df are the degrees of freedom. In progenies with 2 df, the segregation tested hypothesis was 1:2:1 and in other progenies the tested hypothesis was 1:1.

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other alleles that are part of gene regions affected by selection may have been inherited by the progeny. These can have a significant impact on traits for adaptation and breeding of Araucaria. According to BERGMANN et al. (1990), rare alleles in a species, but with intermediate frequency in a population may be important for the performance and adaptability of a population in the long term.

Compared to other studies carried out using microsatellite markers for *A. angustifolia*, our study showed a lower number of alleles for the loci Ag20, Ag23, As90 and CRCAc1, and a greater number of alleles for the loci Aang14 and Aang28. Our results are consistent with most studies for the locus Aang01, which showed the highest number of alleles among all loci (SCHMIDT et al., 2007; STEFENON et al., 2007; SANT'ANNA et al., 2013). However, PATREZE and TSAI (2010) reported that the locus Aang01 had a lower number of alleles than the other five loci used in their study.

No significant deviations from the expected segregation hypothesis (1:1 or 1:2:1) were detected in any of the hand- or open-pollinated progeny sampled (*Tables 4* and *5*). The highest values of the *G*-test were found for the loci Ag45, among the hand-pollinated progenies PF_3 x Gua_1 (G = 5.72) and PF_3 x Lg_Galpão (G = 5.66).

Allele 136 in the locus Aang28 was found only in the male parent PB_1 sampled for hand-pollination and in its progeny. This demonstrates the ability of hand-pollination strategies to form new genotypes, since in a natural setting no hybridization could be accomplished due to the significant distance between the paternal and maternal trees sampled.

In the case of locus Ag23, maternal trees showed a low diversity of alleles. Of the nine genotyped trees with this locus, seven have the same genotype (249/255), except the dioecious tree PF_Solos (247/255) and monoecious tree MN_Ara (249/249). This low polymorphism may be due to sampling, as other studies have found greater variation in maternal trees for this same locus (BITTENCOURT, 2007; SANT'ANNA et al., 2013). Conversely, for locus Ag23, both genotyped (hand-pollinated) and non-genotyped (open-pollinated) pollen donors were important for increasing the number of different alleles in the progenies.

In locus As90, allele 183 was unique in the open-pollinated progeny of the seed tree MN_SD. Although this locus was developed for *Araucaria subulata*, from New Caledonia, it has been shown to be transferable and polymorphic for *A. angustifolia* (BITTENCOURT and SEBBENN, 2007).

The locus CRCAc1 also showed a low number of alleles (three). Only the male parents were found to have allele 203, which again demonstrates the importance of hand-pollination in the formation of new genotypes. We noted that of the nine trees used as maternal parents, only three were heterozygous (PF_Social, PF_1 and MN_Gua) for this locus with genotype 199/201, while the others showed homozygosity with 201/201. BITTENCOURT (2007) also reported that most of the genotyped maternal trees of *A. angustifolia* used in that study were homozygous for the locus CRCAc1, with only two trees showing heterozygosity (200/202). SCOTT et al. (2003) developed the locus CRCAc1 for *Araucaria cunninghamii* and found that the sequences flanking this site are highly preserved and therefore transferable to

Table 5. – Mendelian inheritance in eight microsatellite loci in open-pollinated progenies of *Araucaria angustifolia.*

Locus/Seed tree	Seed tree genotype	*n*	$n_{ij} : n_{ii} - n_{jj}$	$n_{ik} : n_{jk}$	*G*	Locus/Seed tree	Seed tree genotype	*n*	$n_{ij} : n_{ii} + n_{jj}$	$n_{ik} : n_{jk}$	*G*
Ag20						Aang28					
PF_Social	239/241	18	4:2	7:5	0.00	PF_Fazenda	154/162	17	0:0	11:6	1.49
Ag23						PF_Social	150/152	18	0:0	10:8	0.22
PF_Fazenda	249/255	17	3:0	9:5	1.16	Pinha_400	150/152	18	1:0	8:9	0.06
PF_Social	249/255	18	3:0	8:7	0.29	MN_SD	150/168	18	1:7	5:5	0.06
Pinha_400	249/255	18	3:2	6:7	0.07	MN_Gua	152/162	18	4:6	6:2	1.16
MN_SD	249/255	18	5:7	3:3	0.08	As90					
MN_Gua	249/255	18	3:7	5:3	0.60	PF_Fazenda	177/181	17	3:2	7:5	1.16
Ag45						PF_Social	173/175	18	7:3	4:4	0.09
PF_Social	239/241	18	8:10	0:0	0.22	Pinha_400	175/177	18	3:2	7:6	0.60
Aang01						MN_Ara	175/181	18	5:8	3:2	0.08
PF_Fazenda	208/224	17	0:0	8:9	0.06	MN_SD	179/183	18	3:4	7:4	0.07
PF_Social	216/220	18	1:0	10:7	0.53	MN_Gua	175/181	18	3:6	5:4	0.07
Pinha_400	214/216	18	4:1	10:3	4.86	CRCAc1					
MN_Ara	202/214	18	1:6	4:7	1.49	PF_Social	199/201	18	7:3	3:5	2.36
MN_Gua	212/230	18	2:5	2:9	2.31	MN_Gua	199/201	18	7:5	5:1	0.09
Aang14											
PF_Fazenda	162/170	17	0:0	8:9	0.06						
PF_Social	164/170	18	0:0	9:9	0.00						
Pinha_400	150/164	18	1:0	6:11	0.90						
MN_Ara	152/172	18	4:3	3:8	2.66						
MN_SD	152/170	18	4:5	2:7	2.66						
MN_Gua	164/170	18	7:6	4:1	0.83						

n is the sample size. *G* is the value of the maximum likelihood *G*-test for the hypotheses $n_{ij} = n_{ii} + n_{jj}$ and $n_{ik} = n_{jk}$.

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four species of the genus (*A. columnaris*, *A. heterophylla*, *A. luxurians* and *A. bidwillii*) and also for *Aghatis robusta* (Araucariaceae family). According to Scott et al., the low microsatellite variation of this locus suggests its association with gene regions which would be under strong selective pressure against possible mutations. The transferability of locus CRCAc1 to *A. angustifolia* was first verified by Sebbenn and Bittencourt (2007).

Our results confirmed Mendelian inheritance for the eight microsatellite loci tested and no deviations from expected Mendelian segregation (1:1 or 1:2:1) were detected. In all cases tested (110 in total), the progenies of hand- and open-pollination presented low values for the G-test (maximum of 5.72). Thus, these loci can be used without restriction for studies of the mating system, gene flow, genetic diversity and genetic structure of *A. angustifolia*. In contrast, Bittencourt (2007) detected a significant deviation from the expected 1:1 segregation in open-pollinated progenies of two of the eight sampled seed-trees for loci Ag20 and As90, and in two of the 10 maternal trees for the locus Ag23, in a natural population of *A. angustifolia*. Nevertheless, in 42 of 52 cases there was no deviation from the expected segregation.

The small number of detected deviations from Mendelian segregation may be the result of sampling error, small progeny sample size, misinterpretation of the size of alleles, or the presence of null alleles (Hattemer and Gillet, 1989). Genetic factors that cause segregation distortion of significance in molecular markers may be related to pre-zygotic factors that occur between meiosis and gamete fusion and/or post-zygotic factors that occur between fertilization and analysis of the markers. In the first case, three process may occur: (i) meiotic formation occurs with unequal proportions of the types of complementary gametes during meiosis; (ii) selective gametic viability which results in gametic types differing in their ability to survive to fertilization; and (iii) gametic reproductive success which results when gametic types differ in their ability to fertilize the ovules, which, for example, can occur due to gametic self-incompatibility. In the second case, selective mortality related to post-zygotic viability may be occurring between the fertilization stage and the moment we access the genotypes through molecular markers (Gillet and Gregorius, 1992). In this sense, Hufford and Hamrick (2003) found the elimination of homozygous embryos resulting from selfing in *Platypodium elegans* due to inbreeding depression. In the case of the dioecious *A. angustifolia*, the elimination of homozygous seeds may happen due to mating among relatives, causing biparental inbreeding depression.

The eight microsatellite loci used in this study were developed for *A. angustifolia* by Salgueiro et al. (2005) and Schmidt et al. (2007), except for CRCAc1, developed by Scott et al. (2003) for *A. cunninghamii* and As90 developed for *A. subulata*. These authors, as well as Bittencourt (2007) and Sant'Anna et al. (2013) who used these loci to study populations of *A. angustifolia*, detected no significant deviation from Mendelian segregation in these loci after applying the Bonferroni correction. These results support the idea that the loci used herein are not linked in the chromosomes and segregate independently. Thus, these loci can be used together in genetic analyses that require loci that are not linked, such as analyses of mating system and parentage.

Conclusions

The eight microsatellite loci analyzed (Ag20, Ag23, Ag45, Aang01, Aang14, Aang28, As90 and CRCAc1) can be used for genetic studies in *A. angustifolia*, because they present Mendelian segregation in hand- and open-pollinated progeny of monoecious and dioecious trees.

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