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Analysis of Mendelian inheritance and genetic linkage in microsatellite loci of *Eucalyptus urophylla* S.T. Blake

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ABSTRACT. *Eucalyptus urophylla* is an important species in the Brazilian forest sector due to its rapid growth rates and resistance to disease. The aim of this study was to verify Mendelian inheritance, genetic linkage, and genotypic disequilibrium for 15 microsatellite loci, with the goal of producing a robust set of genetic markers. Mendelian inheritance and genetic linkage analyses were carried out using genotypes from maternal trees, and their open-pollinated seeds and genotypic disequilibrium were assessed using adult trees. By comparing heterozygous maternal genotypes and their seeds, we found no significant deviations from the expected 1:1 Mendelian segregation and the expected 1:1:1:1 segregation hypothesis for pairwise loci. For adult trees, we did not find strong evidence of genotypic imbalance for pairwise loci. Our results indicated that the analyzed set of

microsatellite loci could be used to carry out analyses of genetic diversity, mating system, and parentage in *E. urophylla*.

Key words: Eucalypt; Genetic markers; Population genetics; Seed orchards; Tree breeding

INTRODUCTION

The impact of classic quantitative genetics on Brazil's high productivity levels in *Eucalyptus* planted forests is indisputable. However, in the last 25 years, possibilities for further progress have arisen due to technological advancements in molecular markers, sequencing methods, and genetic engineering (Harfouche et al., 2014; Barabaschi et al., 2016). Single sequence repeats (SSR) have been used since 1996 as an auxiliary tool to inform genetic improvement and conservation plans (Byrne et al., 1996), providing essential information on levels of genetic diversity within and among populations, inbreeding, effective population size, breeding system, and gene flow. Among the species cultivated in tropical climate regions, *Eucalyptus urophylla* is planted commercially as a pure species or as a hybrid, mainly with *E. grandis* (Hodge and Dvorak, 2015). The wood can be used in civil construction and pulp and cellulose production (Carvalho et al., 1998). The species is also resistant to diseases such as eucalyptus rust (*Puccinia psidii*), wilt (*Ceratocystis fimbriata*), and cancers (*Chrysoporthe cubensis* and *Coniothyrium zuluense*) (Carvalho et al., 1998).

SSR markers have been used extensively due to their codominant inheritance and high locus polymorphism (Grattapaglia et al., 2012; Randall et al., 2015). However, it is critical to confirm if SSR loci employed in population genetic analyses are in fact genetic markers. These markers cannot suffer from segregation distortions and genetic association between loci, as this would negate the principle of random association between alleles of different loci and generate redundant information. Thus, we assess the Mendelian inheritance, genetic linkage, and genotypic disequilibrium for 15 SSR loci of *E. urophylla* to develop a robust set of genetic markers for genetic diversity, inbreeding, and parentage analyses.

MATERIAL AND METHODS

Two seed orchards of *E. urophylla* (SO1, SO2) and a progeny test (PT) were used for the analyses. The study populations are installed in the Teaching, Research and Outreach Farm (FEPE), Ilha Solteira Faculty of Engineering (FEIS/UNESP), in the municipality of Selvíria, Mato Grosso do Sul State, Brazil. For DNA analysis, we sampled leaves from 79 and 298 adult trees in SO1 and SO2, respectively. In PT, we sampled 605 seedlings (denominated seeds) from 23 mother trees. The extraction and purification of the DNA were performed based on the CTAB method (Doyle and Doyle, 1987) and amplification was performed using PCR (polymerase chain reaction), with minor modifications to the protocol (see Faria et al., 2011). For the genotyping of individuals, we used EMBRA microsatellite markers (Brondani et al., 1998, 2006): 15 SSR loci for SO2 and PT; and 13 common SSR loci for SO1. The genetic characterization was performed in multiplex systems, with multi-fluorescence detection, in an ABI 3100XL automatic capillary sequencer. Raw genotypic data (electropherograms) were exported from the sequencer using the Genotyper software (Applied Biosystems) and adjusted to unity using the TANDEM software (Matschiner and Salzburger, 2009). Molecular analyses were performed at the Hereditas/Genomax Laboratory in Brasília, Brazil.

To investigate the Mendelian inheritance of the SSR loci, we compared maternal heterozygous mother tree genotypes with their seeds, using the method described by Gillet and Hattemer (1989). The assumptions of the method are that the loci have regular segregation and their alleles follow Mendelian inheritance patterns, which are based on the following conditions: i) regular meiotic segregation during ovule production; ii) random fertilization of ovules by each type of pollen; iii) absence of differential selective viability in the progenies prior to the investigation by genetic markers; iv) a co-dominant relationship between alleles. The method further requires that all progeny of a tree possess a maternal allele. In cases of a heterozygous mother tree (e.g., A_iA_j, $i \neq j$), the following are required: a) each one within progeny must have one 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 the number of homozygous progeny A_iA_i (n_{ii}) and A_jA_j (n_{jj}): $n_{ij} = n_{ii} + n_{jj}$; and c) the number of heterozygous progeny A_iA_k (n_{ik}) must be equal to the number of heterozygous progeny A_jA_k (n_{jk}), or $n_{ik} = n_{jk}$, in other words, $k \neq i, j$. The observed segregation of each progeny from a heterozygous maternal tree for a given locus was statistically compared to that expected for the segregation hypothesis of 1:1, using the *G*-test (Sokal and Rohlf, 1981):

$$G = 2\left[n_{ij} \ln\left(\frac{n_{ij}}{E(n1)} \right) + (n_{ii} + n_{ij}) \ln\left(\frac{(n_{ii} + n_{jj})}{E(n1)} \right) \right] \quad \text{(Equation 1)}$$

where ln is the natural logarithm, $E(n1)$ is the expected number of offspring genotypes A_iA_j (n_{ij}) and A_iA_i+A_jA_j(n_{ii}+n_{jj}): $E(n1) = 0.5(n_{ij} + n_{ii} + n_{jj})$, or:

$$G = 2\left[n_{ik} \ln\left(\frac{n_{ik}}{E(n2)} \right) + n_{jk} \ln\left(\frac{n_{jk}}{E(n2)} \right) \right] \quad \text{(Equation 2)}$$

where $E(n2)$ is the expected number of genotypes for alleles A_iA_k (n_{ik}) and A_jA_k (n_{jk}): $E(n2) = 0.5(n_{ik} + n_{jk})$. To avoid false positives, the *G*-test was determined only when $n1$ and $n2$ were ≥15. Deviation from the *G*-test between the observed and expected segregation was determined as statistically significant using the Bonferroni correction for multiple comparisons (95%, $\alpha = 0.05$).

To determine if the loci were genetically linked, we tested pairs of loci using genetic information from mother trees that were doubly heterozygous for two loci (A_iA_j, B_lB_m). The segregation was recorded in their progeny. In this case, the null hypothesis (H_O) was a normal Mendelian segregation of 1:1:1:1. The normal segregation hypothesis between pairs of loci was accepted or rejected based on a maximum likelihood *G*-test (Sokal and Rohlf, 1981) performed for each progeny:

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

where n_{il}, n_{im}, n_{jl}, and n_{jm} are the observed numbers of phenotypes A_iB_l, A_iB_m, A_jB_l, and A_jB_m, respectively, and $E(n)$ is the expected number of each genotype A_iB_l, A_iB_m, A_jB_l, and A_jB_m, calculated by $E(n) = 0.25(n_{il} + n_{im} + n_{jl} + n_{jm})$. Again, we applied the Bonferroni correction for multiple comparisons (95%, $\alpha = 0.05$) to avoid false positives.

The genotypic disequilibrium test between pairwise loci was only performed for adult samples. Estimates of gene frequencies based on open-pollinated progeny arrays are biased because each progeny has at least one maternal allele, resulting in genotypic disequilibrium. This

analysis was carried out using the FSTAT 2.9.3.2 software (Goudet, 2002). The probabilities of the significance test were obtained by permutation of alleles among individuals, associated with a Bonferroni correction for multiple comparisons (95%, α = 0.05).

RESULTS

No deviation from 1:1 Mendelian segregation was detected for 13 of the 15 microsatellite loci (86.6%) analyzed for progenies of heterozygous maternal trees (Table 1). Significant deviations were identified in two cases: EMBRA2 (18.29) for tree 95 and EMBRA63 (12.67) for trees 10 and 155. Of the 733 *G*-tests carried out for pairwise loci, only 26 (3.5%) were significantly different than expected under the hypothesis of 1:1:1:1 independent Mendelian segregation (Table 2). As

Table 1. Mendelian inheritance tests for 15 microsatellite loci in *Eucalyptus urophylla*.

Mother	n_1	$n_{ij}:n_{ii}+n_{jj}$	G_1	n_2	$n_{ik}:n_{jk}$	G_2
EMBRA2						
10, 21, 86	66	26:40	2.99	10	5:5	NE
20, 45	40	18:22	0.04	20	15:5	5.25
55	21	14:7	2.38	6	6:0	NE
95	7	4:3	NE	23	21:2	18.29*
104	17	7:10	0.53	8	8:0	NE
116	23	13:10	0.39	7	3:4	NE
155	10	5:5	NE	20	14:6	3.29
212, 2612	38	13:25	3.86	20	14:6	3.29
214	11	2:9	NE	19	14:5	4.44
EMBRA3						
1	20	9:11	0.20	6	2:4	NE
10, 86, 155	12	6:6	NE	67	46:21	9.56
20, 2612	18	9:9	0	41	19:22	0.22
21, 513	9	0:9	NE	47	23:24	0.02
45	6	1:5	NE	24	15:9	1.52
55	4	2:2	NE	23	16:7	3.62
95	3	1:2	NE	27	19:8	4.61
104, 236	26	11:15	0.62	22	16:6	4.72
116	11	3:8	NE	19	10:9	0.05
EMBRA10						
533	19	9:10	0.05	11	5:6	NE
2612	6	4:2	NE	22	11:11	0
EMBRA11						
10	12	5:7	NE	18	9:9	0
20	15	6:9	0.60	15	10:5	1.70
45, 86	35	22:13	2.34	15	8:7	0.07
55	16	11:5	2.31	11	2:9	NE
EMBRA38						
21	7	0:7	NE	19	11:8	0.48
45	22	11:11	0	8	5:3	NE
55, 65, 95, 101, 104	95	47:46	0.01	37	18:21	0.23
86, 118	14	10:14	NE	25	11:14	0.36
116, 241	42	23:19	0.38	18	7:11	0.90
155	23	9:14	1.10	7	5:2	NE
212	2	0:2	NE	27	10:17	1.84
214	18	5:13	3.68	10	5:5	NE
EMBRA63						
10, 155	35	11:24	4.95	25	4:21	12.67*
21, 45, 101, 118	103	65:38	7.16	13	10:3	NE
55	21	7:14	2.38	6	2:4	NE
95. 241	46	27:19	1.40	14	10:4	NE
104	6	1:5	NE	19	11:8	0.48
2612	21	8:13	1.20	7	2:5	NE
EMBRA128						
1, 116, 214	57	21:36	3.99	27	14:13	0.04
10	6	0:6	NE	24	9:15	1.52
21	16	4:12	4.19	10	4:6	NE
118	11	3:8	NE	19	4:15	6.78
212	23	5:18	7.80	7	4:3	NE
EMBRA157						
20, 45, 118, 212	72	23:49	9.60	47	23:24	0.02
21, 116	49	16:33	6.02	7	2:5	NE
65	17	4:13	5.02	2	0:2	NE
95	6	3:3	NE	24	17:7	4.30
458	11	1:10	NE	19	8:11	0.48
EMBRA219						
1, 101	45	24:21	0.20	11	8:3	NE
10	13	4:9	NE	17	7:10	0.53
20, 21, 55, 116, 155, 2612	96	34:62	8.29	75	33:42	1.08
45	17	12:5	2.97	13	4:9	NE
95	13	7:6	NE	17	6:11	1.49
118	15	7:8	0.07	15	6:9	0.60
214	12	4:8	NE	18	9:9	0
236	15	8:7	0.07	8	4:4	NE
241	22	8:14	1.66	8	5:3	NE
458	16	6:10	1.01	14	11:3	NE
EMBRA333						
1	20	14:6	3.29	5	3:2	NE
10	22	6:16	4.72	8	3:5	NE
21	15	7:8	0.07	14	6:8	NE
45, 2612	29	11:18	1.71	29	17:12	0.87
101	23	16:7	3.62	7	5:2	NE
116	11	2:9	NE	20	10:10	0
155	19	9:10	0.05	11	11:0	NE
214	7	0:7	NE	23	13:10	0.39
241	22	9:13	0.73	8	2:6	NE
53+000000000000000000 00000000000000000000 00000000000000000000 00000000000000000000 0000000003	24	10:14	0.67	6	0:6	NE

Mother	n_1	$n_{ij}:n_{ii}+n_{jj}$	G_1	n_2	$n_{ik}:n_{jk}$	G_2
EMBRA12						
10, 212	18	11:7	0.90	41	14:27	4.19
20	11	6:5	NE	19	11:8	0.48
45	18	10:8	0.22	12	7:5	NE
55	19	8:11	0.48	8	2:6	NE
86, 95, 458, 2473	50	24:26	0.08	43	24:19	0.58
101	26	16:10	1.40	4	0:4	NE
104	7	5:2	NE	18	13:5	3.68
116	18	10:8	0.22	12	3:9	NE
118	11	8:3	NE	17	4:13	5.02
155, 2612	25	18:7	5.01	31	14:17	0.29
214	6	2:4	NE	24	10:14	0.67
533	11	8:3	NE	19	6:13	2.64
EMBRA28						
1	19	9:10	0.05	6	5:1	NE
10	12	6:6	NE	18	4:14	5.88
21	2	1:1	NE	24	8:16	2.72
45	19	12:7	1.33	11	2:9	NE
55	4	3:1	NE	23	10:13	0.39
101	22	15:7	2.98	8	7:1	NE
104	7	3:4	NE	18	11:7	0.90
116	11	6:5	NE	19	8:11	0.48
155	17	5:12	2.97	13	8:5	NE
212	2	0:2	NE	28	17:11	1.30
214	10	0:10	NE	20	12:8	0.80
236	6	2:4	NE	17	9:8	0.06
241	13	6:7	NE	16	8:8	0
458	9	4:5	NE	21	13:8	1.20
EMBRA157						
2612	18	4:14	5.88	9	4:5	NE
EMBRA204						
10	18	6:12	2.04	12	7:5	NE
20, 2612	30	10:20	3.40	30	12:18	1.21
21	5	4:1	NE	21	10:11	0.05
45	23	7:16	3.62	7	4:3	NE
95, 212	11	6:5	NE	48	17:31	4.14
116	11	8:3	NE	19	6:13	2.64
118	18	10:8	0.22	12	12:0	NE
155	19	7:12	1.33	11	6:5	NE
214, 533	36	17:19	0.11	24	18:6	6.28
458	6	2:4	NE	24	9:15	1.52
EMBRA210						
21	8	5:3	NE	19	8:11	0.48
45	18	12:6	2.04	13	7:6	NE
55	7	5:2	NE	20	9:11	0.20
95	5	3:2	NE	23	8:15	2.16
116	9	2:7	NE	22	7:15	2.98
118	19	10:9	0.05	11	4:7	NE
155	14	7:7	NE	16	9:7	0.25
212	13	10:3	NE	15	6:9	0.60
214	18	9:9	0	12	12:0	NE
533	20	12:8	0.80	10	4:6	NE
EMBRA681						
10	11	2:9	NE	19	12:7	1.33
45	19	13:6	2.64	11	3:8	NE
116	12	3:9	NE	18	10:8	0.22
118	15	8:7	0.07	15	12:3	5.78
155	20	12:8	0.80	10	1:9	NE
212	15	8:7	0.07	14	9:5	NE
533	15	7:8	0.07	14	8:6	NE
2612	9	3:6	NE	19	10:9	0.05

n_1 and n_2 are the sample size; G_1 and G_2 are the maximum likelihood *G* statistics for the hypothesis of $n_{ij}:n_{ii}+n_{jj}$ and $n_{ik}:n_{jk}$, respectively, for one degree of freedom. *Significance after Bonferroni correction for α = 0.05 (χ^2 = 10.83). NE is not estimated due to a sample size of less than 15 progeny.

Table 2. Values of maximum likelihood *G*-test for the hypothesis of independent segregation between pairwise loci (1:1:1:1) for *Eucalyptus urophylla*.

Mother	*G*	Mother	*G*	Mother	*G*	Mother	*G*	Mother	*G*
EMBRA2xEMBRA28		EMBRA2xEMBRA11		EMBRA2xEMBRA63		EMBRA28xEMBRA3		EMBRA28xEMBRA10	
10	12.04	55	2.96	2612	5.61	214	0.58	55	0.80
21	3.97	86	1.46	EMBRA2xEMBRA12		236	1.26	86	0.95
45	5.26	104	9.28	10	2.87	241	5.33	101	2.31
55	0.25	116	3.70	20	3.58	458	0.24	104	8.73
86	3.19	155	6.67	21	2.46	2473	1.27	116	0.05
104	13.72	212	2.19	45	2.02	EMBRA28xEMBRA11		155	2.95
116	2.04	2612	4.27	55	6.15	1	2.82	212	1.07
155	6.43	EMBRA2xEMBRA10		86	2.60	10	11.05	214	23.68*
212	3.05	10	10.90	95	20.79*	21	1.87	236	9.23
214	4.69	20	7.50	104	10.58	45	3.82	241	4.34
EMBRA2xEMBRA3		21	3.67	116	3.61	55	1.30	458	1.93
10	9.57	45	2.78	155	7.48	86	0.30	2473	0.67
20	2.69	55	1.65	212	4.46	101	1.37	EMBRA28xEMBRA63	
21	2.69	86	5.60	214	5.99	104	3.31	10	31.82*
45	1.30	104	5.19	2612	1.79	116	3.00	21	4.23
55	8.02	155	7.09	EMBRA28xEMBRA3		155	4.53	45	3.61
86	6.62	212	1.94	1	1.37	212	1.76	55	6.34
95	21.19*	214	NE	10	11.57	214	0.58	101	0.95
104	16.94*	2612	1.51	21	5.20	236	9.74	104	4.34
116	1.19	EMBRA2xEMBRA63		45	3.89	241	4.36	155	15.37
155	7.77	10	19.51*	55	3.32	458	4.06	241	4.38
2612	1.98	21	5.54	86	2.89	2473	0.12	2473	5.27
EMBRA2xEMBRA11		45	4.89	101	1.11	EMBRA28xEMBRA10		EMBRA28xEMBRA12	
10	0.49	55	9.26	104	2.85	1	2.42	10	7.74
20	3.78	95	20.39*	116	1.53	10	11.97	21	6.28
21	2.43	104	5.36	155	5.44	21	7.59	45	3.38
45	1.17	155	10.62	212	1.62	45	3.03	55	1.96
Mother	*G*	Mother	*G*	Mother	*G*	Mother	*G*	Mother	*G*
EMBRA28xEMBRA12		EMBRA3xEMBRA11		EMBRA3xEMBRA63		EMBRA3xEMBRA157		EMBRA11xEMBRA10	
86	0.20	86	1.48	45	6.57	21	3.18	55	2.97
101	0.49	104	10.39	55	5.56	45	2.16	65	0.88
104	2.61	116	2.40	95	7.34	65	0.67	86	3.25
116	0.37	155	17.28*	104	3.28	95	7.46	101	3.08
155	1.62	236	13.57	155	11.04	116	2.60	104	5.55
212	4.15	533	4.41	2473	3.23	236	7.90	155	2.47
214	0.79	2612	5.44	2612	1.74	2473	0.44	212	0.96
236	6.06	EMBRA3xEMBRA10		EMBRA3xEMBRA12		2612	2.57	236	15.98
458	2.87	1	0.73	10	4.27	EMBRA3xEMBRA204		241	2.11
2473	0.29	10	5.88	20	0.28	10	3.49	458	3.98
EMBRA28xEMBRA157		20	8.10	21	2.22	20	10.22	533	0.18
20	4.07	21	6.25	45	1.03	21	5.85	2612	1.79
45	3.01	45	6.95	55	5.27	45	4.59	EMBRA11xEMBRA63	
116	3.52	55	6.46	65	0.73	55	4.56	10	18.32*
212	4.58	65	0.45	86	4.72	86	9.77	21	8.69
236	5.15	86	5.94	95	8.26	95	8.17	45	4.79
458	2.70	104	2.60	104	5.89	116	1.43	55	11.37
2473	1.07	155	8.57	116	1.34	155	5.83	101	3.54
EMBRA3xEMBRA11		236	10.65	155	4.91	533	3.57	104	4.54
10	4.58	533	3.79	236	3.17	2612	5.20	118	1.65
20	0.07	2473	1.26	533	5.56	EMBRA11xEMBRA10		155	12.79
21	7.03	2612	0.80	2473	0.29	10	6.98	241	4.60
45	2.92	EMBRA3xEMBRA63		2612	1.12	20	6.88	2612	3.52
55	4.47	10	18.98*	EMBRA3xEMBRA157		21	6.79	EMBRA11xEMBRA12	
65	0.08	21	7.61	20	4.20	45	2.64	10	13.37
Mother	*G*	Mother	*G*	Mother	*G*	Mother	*G*	Mother	*G*
EMBRA11xEMBRA12		EMBRA11xEMBRA157		EMBRA11xEMBRA219		EMBRA10xEMBRA12		EMBRA10xEMBRA204	
20	0.38	458	2.61	116	4.63	86	1.35	45	3.00
21	4.83	2612	0.53	118	6.34	101	2.13	55	2.75
45	0.81	EMBRA11xEMBRA204		155	0.88	104	1.83	86	9.11
55	3.33	10	1.96	236	10.04	155	3.49	155	1.73
65	0.62	20	1.00	241	0.69	212	5.04	212	3.87
86	3.82	21	2.83	458	11.61	214	NE	214	36.71*
101	2.87	45	1.79	2612	5.32	236	17.39*	458	4.86
104	1.36	55	2.03	EMBRA10xEMBRA63		458	8.66	533	2.43
116	15.08	86	6.09	10	30.24*	533	1.72	2612	0.80
118	10.56	116	5.34	21	8.80	2473	1.80	EMBRA10xEMBRA219	
155	4.43	118	7.17	45	8.03	2612	4.93	1	1.22
212	4.85	155	2.28	55	5.32	EMBRA10xEMBRA157		10	9.87
236	10.07	212	7.16	101	3.04	20	4.23	20	11.29
458	4.85	458	2.52	104	0.88	21	3.28	21	4.65
533	1.87	533	1.26	155	19.15*	45	1.57	45	9.38
2612	2.61	2612	0.84	241	6.93	65	0.98	55	3.26
EMBRA11xEMBRA157		EMBRA11xEMBRA219		2473	2.23	212	5.39	65	6.12
20	0.73	10	2.37	2612	2.15	236	22.45*	86	1.97
21	7.87	20	2.58	EMBRA10xEMBRA12		458	1.66	101	9.37
45	1.26	21	6.99	10	6.34	2473	0.57	155	4.56
65	0.90	45	3.00	20	6.01	2612	0.83	214	23.30*
116	3.08	55	2.99	21	3.89	EMBRA10xEMBRA204		236	12.52
118	2.69	65	2.16	45	0.84	10	7.03	241	5.56
212	6.05	86	0.13	55	1.19	20	4.70	458	8.80
236	16.23	101	3.42	65	0.91	21	9.32	2473	5.60
Mother	*G*	Mother	*G*	Mother	*G*	Mother	*G*	Mother	*G*
EMBRA10xEMBRA219		EMBRA63xEMBRA12		EMBRA63xEMBRA219		EMBRA12xEMBRA157		EMBRA12xEMBRA219	
2612	3.89	155	12.93	101	1.97	116	3.03	20	20.13*

Continued on next page

Table 2. Continued.

EMBRA10xEMBRA333		2473	4.73	118	11.98	118	2.40	21	5.18
1	2.96	2612	2.19	155	11.65	212	7.99	45	1.74
10	17.32*	EMBRA63xEMBRA157		241	5.40	236	13.02	55	2.19
21	6.59	21	8.90	2473	3.32	458	2.28	65	3.37
45	5.34	45	4.60	2612	4.22	2473	0.12	86	0.34
65	2.94	95	8.23	EMBRA63xEMBRA333		2612	1.74	95	0.20
86	6.40	118	0.83	10	25.43	EMBRA12xEMBRA204		101	2.87
101	2.76	2473	5.69	21	9.59	10	0.33	116	2.85
155	9.21	2612	0.80	45	1.26	20	2.01	118	4.53
212	5.15	EMBRA63xEMBRA204		101	0.78	21	1.38	155	2.03
214	NE	10	8.50	155	16.11	45	1.46	214	0.88
241	6.11	21	2.86	241	13.84	55	1.72	236	1.13
533	2.03	45	8.61	2473	1.54	86	10.84	458	7.21
2473	1.01	55	5.17	2612	4.46	95	1.40	2473	4.72
2612	3.35	95	7.20	EMBRA63xEMBRA128		116	3.42	2612	1.48
EMBRA63xEMBRA12		118	7.45	10	15.54	118	11.78	EMBRA12xEMBRA333	
10	21.92*	155	15.17	21	6.69	155	1.74	10	1.48
21	3.75	2612	0.76	118	13.48	212	8.09	21	1.35
45	5.14	EMBRA63xEMBRA219		EMBRA12xEMBRA157		214	6.28	45	0.73
55	3.81	10	20.57*	20	1.23	458	5.48	65	5.17
95	6.59	21	4.48	21	1.36	533	2.71	86	4.44
101	2.35	45	6.00	45	0.06	2612	1.59	101	0.74
104	5.96	55	5.83	65	3.05	EMBRA12xEMBRA219		116	8.19
118	3.77	95	2.75	95	4.06	10	3.35	155	10.24
Mother	*G*	Mother	*G*	Mother	*G*	Mother	*G*	Mother	*G*
EMBRA12xEMBRA333		EMBRA12xEMBRA38		EMBRA157xEMBRA333		EMBRA157xEMBRA210		EMBRA204xEMBRA333	
212	9.01	212	10.26	21	1.41	95	3.97	212	9.77
214	4.18	214	1.97	45	3.04	116	3.43	214	7.01
533	2.95	236	2.42	65	2.41	118	0.25	533	0.72
2473	1.54	EMBRA157xEMBRA204		116	5.48	212	10.00	2612	0.49
2612	10.73	20	4.01	212	5.92	EMBRA204xEMBRA219		EMBRA204xEMBRA128	
EMBRA12xEMBRA128		21	2.33	2473	1.02	10	1.23	10	3.21
10	2.08	45	2.37	2612	1.95	20	3.21	21	3.37
21	2.32	95	1.79	EMBRA157xEMBRA128		21	8.97	86	16.49*
86	1.66	116	1.44	21	5.38	45	9.77	116	6.20
116	2.62	118	4.23	116	1.98	55	2.02	118	6.62
118	17.19*	212	9.66	118	12.65	86	5.75	212	7.07
212	3.39	458	2.79	212	5.19	95	3.81	214	16.94*
214	12.83	2612	0.90	EMBRA157xEMBRA38		116	3.95	EMBRA204xEMBRA38	
EMBRA12xEMBRA38		EMBRA157xEMBRA219		21	3.25	118	10.97	21	1.74
21	7.83	20	0.56	45	0.47	155	3.04	45	5.21
45	0.57	21	1.53	65	1.10	214	12.61	55	0.51
55	0.73	45	4.01	95	5.94	458	9.77	86	11.22
65	0.31	65	2.89	116	1.72	2612	4.03	95	1.23
86	1.50	95	7.21	118	0.48	EMBRA204xEMBRA333		116	2.53
95	0.83	116	3.69	212	3.35	10	6.24	118	4.49
101	1.52	118	6.99	236	16.86*	21	3.15	155	3.23
104	6.68	236	10.03	EMBRA157xEMBRA210		45	4.14	212	6.81
116	1.52	458	16.27*	21	2.33	86	9.68	214	5.84
118	8.16	2473	3.76	45	0.67	116	5.33	EMBRA204xEMBRA210	
155	3.71	2612	4.47	65	4.59	155	10.75	21	8.72
Mother	*G*	Mother	*G*	Mother	*G*	Mother	*G*	Mother	*G*
EMBRA204xEMBRA210		EMBRA219xEMBRA333		EMBRA219xEMBRA38		EMBRA219xEMBRA681		EMBRA333xEMBRA210	
45	3.72	45	3.50	101	4.33	118	7.99	45	0.97
55	12.14	65	6.22	116	4.60	155	4.24	65	6.85
86	5.29	86	4.17	118	7.82	2473	4.86	86	2.83
95	3.38	101	1.11	155	3.86	2612	9.10	116	13.28
116	7.97	116	3.25	214	5.19	EMBRA333xEMBRA128		155	5.24
118	8.56	155	11.38	236	0.23	1	0.30	212	7.84
155	3.53	214	9.03	241	1.57	10	5.04	214	11.37
212	8.48	241	2.50	EMBRA219xEMBRA210		21	2.81	533	0.90
214	14.94	2473	2.08	21	8.97	86	4.64	EMBRA333xEMBRA681	
533	1.74	2612	4.66	45	3.17	116	2.08	10	5.42
EMBRA204xEMBRA681		EMBRA219xEMBRA128		55	10.09	212	2.32	45	0.75
10	1.53	1	1.18	65	6.91	214	12.75	65	7.75
45	2.92	10	5.12	86	3.55	EMBRA333xEMBRA38		116	3.49
55	1.14	21	9.14	95	2.87	21	0.31	155	7.80
95	11.18	86	2.65	116	13.76	45	6.35	212	3.27
116	3.46	116	4.71	118	3.65	65	3.60	533	0.38
118	12.25	118	16.13	155	3.18	86	6.00	2473	4.32
155	1.05	214	12.12	214	13.16	101	1.80	2612	6.44
212	1.68	EMBRA219xEMBRA38		EMBRA219xEMBRA681		116	7.35	EMBRA128xEMBRA38	
533	0.07	21	2.56	10	1.40	155	8.86	21	5.58
2612	4.66	45	3.25	45	2.09	212	8.26	86	1.88
EMBRA219xEMBRA333		55	1.74	55	0.65	214	3.84	116	1.87
1	0.20	65	2.12	65	4.36	241	4.30	118	6.59
10	5.89	86	0.39	95	11.98	EMBRA333xEMBRA210		212	3.72
21	5.74	95	1.20	116	3.36	21	1.90	214	12.34
Mother	*G*	Mother	*G*	Mother	*G*	Mother	*G*	Mother	*G*
EMBRA128xEMBRA210		EMBRA128xEMBRA681		EMBRA38xEMBRA210		EMBRA38xEMBRA681		EMBRA210xEMBRA681	
21	4.44	118	16.97*	116	13.66	95	12.36	95	9.33
86	3.44	212	1.73	118	3.17	116	0.75	116	6.97
116	8.44	EMBRA38xEMBRA210		155	1.30	118	5.13	118	2.88
118	11.07	21	1.74	212	5.69	155	2.57	155	1.46
212	2.35	45	0.27	214	10.27	212	4.49	212	1.27
214	15.41	55	0.53	EMBRA38xEMBRA681		EMBRA210xEMBRA681		533	0.06
EMBRA128xEMBRA681		65	5.31	45	0.58	45	0.53		
10	0.34	86	2.38	55	0.52	55	0.90		
116	1.15	95	2.08	65	2.42	65	8.47		

*Significance after Bonferroni correction for $\alpha = 0.05$ ($\chi^2 = 16.27$). *G* is the *G*-test at three degrees of freedom.

the deviations were observed in different families, our results do not provide solid evidence of genetic linkage between the loci (Table 3). Considering the two seed orchards, only 5.8% of the pairwise loci were significant for genotypic disequilibrium. In both orchards, significance was found between the following loci: EMBRA681 x EMBRA2 and EMBRA204 x EMBRA210.

Table 3. Genotypic disequilibrium between pairwise microsatellite loci for *Eucalyptus urophylla* adult trees from seed orchard SO1 (lower diagonal) and SO2 (upper diagonal).

	EMB2	EMB28	EMB3	EMB11	EMB10	EMB63	EMB12	EMB157	EMB204	EMB128	EMB38	EMB210	EMB681
EMB2	-	0.01090	0.00363	0.08419	0.37607	0.05406	0.16368	0.33718	0.32885	0.09829	0.31346	0.01068	0.00021*
EMB28	0.55449	-	0.07778	0.13120	0.12308	0.01111	0.00363	0.02308	0.07863	0.01239	0.17073	0.05449	0.13312
EMB3	0.01731	0.00278	-	0.08205	0.00385	0.00983	0.00128	0.00021*	0.00043	0.00812	0.00705	0.00192	0.03547
EMB11	0.66175	0.02970	0.06175	-	0.00406	0.08462	0.00299	0.31880	0.30299	0.14124	0.16453	0.12991	0.00641
EMB10	0.93462	0.48397	0.80321	0.45855	-	0.00556	0.10299	0.49017	0.11432	0.08868	0.00021*	0.02393	0.34979
EMB63	0.03718	0.18974	0.00534	0.27030	0.26538	-	0.27842	0.01987	0.22628	0.69380	0.04359	0.10043	0.08419
EMB12	0.14615	0.66731	0.04487	1.0000	0.08718	0.40342	-	0.00299	0.03376	0.11346	0.06132	0.04850	0.02778
EMB157	0.64017	0.10192	0.00919	0.00620	0.26410	0.06731	0.02885	-	0.02073	0.05000	0.02009	0.00256	0.01239
EMB204	0.01667	0.85128	0.03120	0.36731	0.76154	0.18568	0.85491	0.30855	-	0.13654	0.00171	0.00021*	0.00021*
EMB128	0.13889	0.00620	0.15085	0.03868	0.23974	0.21859	0.33226	0.39487	0.87286	-	0.00021*	0.18483	0.06944
EMB38	0.58932	0.30342	0.04444	0.56731	0.25833	0.88739	0.62115	0.75021	0.31346	0.27286	-	0.05983	0.00150
EMB210	0.06709	0.84167	0.02970	0.33739	0.53462	0.27970	0.83504	0.30726	0.00021*	0.86239	0.26560	-	0.00021*
EMB681	0.00021*	0.04103	0.10021	0.06261	0.04231	0.14530	0.93568	0.13355	0.64316	0.06026	0.71816	0.38590	-

P values represent the probability of genotypic disequilibrium after 1440 permutations of alleles among individuals. Value at which results are deemed significant after Bonferroni correction: *P = 0.00021 ($\alpha = 0.05$).

DISCUSSION

Mendelian 1:1 segregation at the individual locus was confirmed for 13 of the tested SSR loci. Significant deviations were only found in isolated cases of EMBRA2 (tree 95) and EMBRA63 (trees 10 and 155), where we found disproportionate results for segregated maternal alleles in their progenies. Of the 30 seeds collected from mother tree 95 (110/132), 27 received allele 110 (90%); for trees 10 and 155 (168/172), 30 (100%), and 26 (86.7%) of the 30 offspring, respectively, received allele 172. These results suggest the occurrence of segregation deviation caused by pre- or post-zygotic factors. Segregation deviations in a limited number of SSR loci of some families can also be caused by sampling errors, small family size, misinterpretation of allele size, or the presence of null alleles (Danner et al., 2013; Tambarussi et al., 2013). Nevertheless, these limited instances found herein do not indicate that the loci deviate from the expected Mendelian inheritance and we can conclude that the 15 EMBRA SSR markers are genetic markers.

The small numbers of significant *G*-test deviations from independent segregation between pairs of loci (1:1:1:1) indicate that the loci segregate independently. Significant values were observed for different families and occurred more frequently between the following pairs: EMBRA2xEMBRA3, EMBRA2xEMBRA63, and EMBRA204xEMBRA128. The significant linkage may be the result of true genetic linkage or deviations from 1:1 Mendelian segregation (Manoel et al., 2015; Moraes et al., 2015), as observed for EMBRA2 and EMBRA63. The absence of linkage between the loci is important concerning models used in population genetic analyses, which assume random segregation between alleles of different loci.

Considering the two seed orchards, our results do not indicate genotypic disequilibrium. Only 5.8% of the results between pairs of loci were significant, and the majority (78%) was detected in SO2. This is likely due to relatedness that exists between the 79 and 298 adult trees on SO1 and SO2, respectively, and the unbalanced proportion of individuals within families that remained after selective thinning during orchard establishment. In both orchards, significance was found between loci EMBRA681xEMBRA2 and EMBRA204xEMBRA210. The imbalance may be affected by selection, recombination, migration, population reduction,

genetic drift, and population structure (Kumar et al., 2004). Tarazi et al. (2010) found genotypic disequilibrium between pairwise loci in open-pollinated seeds of *Copaifera langsdorffii*, probably due to the inheritance of maternal alleles. When the family structure was considered in the analysis, with a limited number of seeds per family, a few significant values of genotypic disequilibrium were detected, thus supporting the idea that the inheritance of maternal alleles produces genotypic disequilibrium. In conclusion, our results show that the 15 SSR loci analyzed herein form a robust set of genetic markers, which can be used to assess issues related to genetic diversity, mating system, and parentage analysis, providing more in-depth information that can be used to advance *E. urophylla* breeding programs.

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