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Mendelian inheritance, genetic linkage, and genotypic disequilibrium at microsatellite loci in *Genipa americana* L. (Rubiaceae)

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ABSTRACT. *Genipa americana* is a tropical tree species that is widely distributed in the humid tropical and subtropical regions of Central and South America. This study investigated Mendelian inheritance, genetic linkage, and genotypic disequilibrium at six microsatellite loci developed for *G. americana*. Adult trees (188) and regenerants (163) were sampled and genotyped in a fragmented population of the species. We also genotyped open-pollinated seeds from 12 seed-trees during reproductive events in 2010 and 2011. Significant deviations from the expected 1:1 Mendelian segregation were detected in 29.5% of the tests. Significant genetic linkage between pairwise loci was detected

in 54.4% of the tests, but no genotypic disequilibrium was detected between pairwise loci for adult trees and regenerants. Overall, the results indicate that the six loci analyzed may be used in studies of *G. americana*'s genetic diversity and structure, its mating system, and in parentage analyses.

Key words: Conservation; Jenipapo; Microsatellites; Tropical tree species; Population genetics

INTRODUCTION

Genipa americana L. (Rubiaceae) is a dioecious tropical tree species, with pollination by bees and wasps and fruit dispersal by gravity, zoochory, and hydrochory (Carvalho, 1994). The tree is heliophytic, semideciduous, and selectively hygrophytic, and is economically important for wood and food production. The species occurs naturally in temporarily or permanently flooded areas of open forests and floodplains, in tropical and subtropical regions of Latin America (Carvalho, 1994). However, due to the intense fragmentation that its habitat has suffered, in isolated forest remnants only a few individuals of the species remain. Therefore, its genetic conservation has become a priority that only can be achieved through studies of its genetic diversity and structure, gene flow, and mating system. For these studies, microsatellite markers (simple sequence repeats, SSRs) have been used, due to their high degree of polymorphism in terms of the number of alleles (Ashley, 2010). However, if SSRs are developed for a species to be used as genetic markers, the criteria include the loci being linked and that inheritance follows Mendelian segregation rules. In addition, equilibrium linkage should be investigated as it can be altered due to selfing, correlated mating, mating between related individuals, and factors such as genetic drift and bottlenecks, which result from forest fragmentation and logging, as well as from founder events.

In the present study, we investigated Mendelian inheritance, genetic linkage, and genotypic disequilibrium at six microsatellite loci developed for *G. americana* by Manoel et al. (2014).

MATERIAL AND METHODS

Open-pollinated seeds were collected from the canopy of 25 seed-trees randomly selected in the Mata da Figueira, a forest fragment belonging to the Mogi Guaçu Ecological Station (22°22'02"S, 51°25'08"W) during reproductive events in 2010 (13 seed-trees) and 2011 (12 seed-trees). The seeds were germinated separately for each seed-tree, and 27 to 80 seeds were obtained per tree. Cambial tissue from 188 adult trees and leaf tissue from 163 regenerants was also collected for genetic analysis.

DNA was extracted from the cambial tissue of all 188 adult trees using the method

of Novaes et al. (2009). Progeny and regenerant DNA was extracted from the leaf tissue of germinated seeds using the method of Doyle and Doyle (1987).

Six primers were used (Manoel et al., 2014). Microsatellite loci were amplified by polymerase chain reaction in a 15-mL final volume, using GoTaq® Colorless Master Mix containing 7.5 mL 2X GoTaq® Colorless Master Mix, 0.9 mM of each primer (forward and reverse), 3.0 µL nuclease-free water, and 7.5 ng template DNA. The amplification program for all the primers consisted of an initial denaturing step at 94°C for 1 min, followed by 35 cycles of each amplification at 94°C for 1 min, 1 min at the specific annealing temperature for each primer pair (Manoel et al., 2014), 72°C for 1 min, and a final elongation step at 72°C for 10 min. Amplifications were performed using a Mastercycler (Eppendorf, Hamburg, Germany). The amplification products (2-mL total reaction volume) were separated on a Fragment Analyzer™ Automated CE System [Advanced Analytical Technologies Inc. (AATI), Ames, IA, USA] using a dsDNA Reagent Kit, 35-500 bp (DNF-900, Advanced Analytical Technologies Inc.). Raw data were analyzed using the PROSize™ (version 2.0) software (AATI).

The method developed by Gillet and Hattemer (1989) was used to investigate the Mendelian inheritance of the *G. americana* SSR loci, which is based on comparisons of a heterozygous maternal genotype tree with the segregation of its alleles in an open-pollinated progeny. This method assumes that the loci have regular segregation and that their alleles follow classic Mendelian inheritance patterns, which are based on three main requirements: i) regular meiotic segregation during ovule production; ii) random ovule fertilization by a type of pollen; iii) no selection between the moment of fertilization and the genotyping of the seeds. The model also assumes that there is a co-dominant relationship between all of the alleles. The method further requires that the following conditions are met: all the progeny of a tree must possess a maternal allele, and in cases of heterozygous parent trees (e.g., A_iA_j, $i \neq j$): a) each individual 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 the 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 $k \neq i, j$.

Using this model and the open-pollinated progenies sampled from 25 seed-trees in two reproductive events, we proceeded to compare the segregation observed in each progeny of the heterozygous maternal tree for a given loci, with the expectation of a classic Mendelian 1:1 segregation, using a G-test (Sokal and Rohlf, 1981):

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

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:

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

where *ln* is the natural logarithm and *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's correction for multiple comparisons (95%, $\alpha = 0.05$) was used to avoid false positives.

To test whether the loci 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; we recorded the segregation in their progeny. In this case, the null hypothesis (H_O) was the classic Mendelian 1:1:1:1 segregation. The hypothesis of classic segregation between the pairwise loci was accepted or discarded based on a maximum likelihood G-test (Sokal and Rohlf, 1981) that was conducted 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_{jl} are the observed number of phenotypes A_iB_k, A_iB_l, A_jB_k, and A_jB_l, respectively, and $E(n)$ is the expected number of genotypes A_iB_k, A_iB_l, A_jB_k, and A_jB_l, and *ln* is the natural logarithm. $E(n)$ was calculated as $E(n) = 0.25(n_{ik} + n_{il} + n_{jk} + n_{jl})$. A Bonferroni's correction for multiple comparisons (95%, $\alpha = 0.05$) was also applied.

A genotypic disequilibrium test was conducted for the adult trees and regenerants, since genotypic disequilibrium is expected in progeny arrays because all of the descendants always receive a maternal allele. The genotypic disequilibrium test was carried out using the FSTAT program (Goudet, 2002) that included a Bonferroni's correction (95%, $\alpha = 0.05$).

RESULTS

After the Bonferroni's correction, the results showed significant deviations from the expected 1:1 Mendelian segregation pattern in 41 cases (29.5%) of 139 tests (Tables 1 and 2), and 74 of the 136 linkage tests (54.4%) were significant (Table 3), suggesting that a physical linkage was present between some pairwise loci. In general, linkage occurred between different pairs of loci of different progenies, and between all of the progenies for the Gam01x-Gam24 pair of loci. However, after the Bonferroni's correction, no significant genotypic disequilibrium between pairs of loci in the adults and regenerants was detected, suggesting that a state of linkage equilibrium existed (Table 4).

Table 1. Mendelian inheritance tests of three microsatellite loci of *Genipa americana*.

Seed-tree	Genotype	n_1	$n_{ij} : n_i + n_j$	G_1 (GL = 1)	n_2	$n_{ik} : n_{jk}$	G_2 (GL = 1)
Gam01							
8	170/192	75	6:69	62.16*	5	3:2	NE
9	170/192	34	0:34	NE	45	38:7	23.48*
14	170/176	44	13:31	7.58	36	35:1	40.77*
21	170/192	26	9:17	2.50	2	2:0	NE
24	170/186	59	42:17	10.93	18	12:6	2.04
25	170/176	32	12:20	2.02	39	8:31	14.49*
26	170/182	54	16:38	9.22	26	26:0	NE
28	160/176	33	10:23	5.26	46	28:18	2.19
35	170/176	36	8:28	11.76	44	43:1	51.45*
37	160/170	47	16:31	4.87	26	1:25	27.57*
38	170/192	37	1:36	42.09*	43	43:0	NE
39	170/176	27	1:26	28.88*	44	27:17	2.29
79	160/176	59	18:41	9.21	16	7:9	0.25
85	170/186	24	7:17	4.29	55	27:28	0.02
107	142/160	37	13:24	3.32	21	12:9	0.43
128	160/172	43	31:12	8.69	31	2:29	28.14*
129	156/172	52	32:20	2.79	7	3:4	NE
145	170/176	42	22:20	0.10	13	1:12	10.97
147	160/176	47	20:27	1.05	26	1:25	27.57*
158	160/176	39	23:16	1.26	19	4:15	6.78
161	176/196	36	12:24	4.08	29	26:3	20.91*
Gam02							
8	164/180	76	28:48	5.33	4	3:1	NE
9	164/180	74	30:44	2.66	4	2:2	NE
14	188/208	76	43:33	1.32	0	0:0	NE
21	164/180	25	14:11	0.36	5	2:3	NE
24	164/180	31	14:17	0.29	45	19:26	1.09
25	158/180	76	48:28	5.33	3	2:1	NE
26	180/226	61	39:22	4.80	18	10:8	0.22
29	164/208	49	26:23	0.18	31	4:27	19.13*
37	162/180	57	33:24	1.43	21	0:21	NE
39	164/188	57	26:31	0.44	22	14:08	1.66
79	164/188	56	37:19	5.89	20	0:20	NE
107	164/180	70	43:27	3.69	7	5:2	NE
128	164/180	71	37:34	0.13	9	6:3	NE
129	158/174	67	30:37	0.73	9	6:3	NE
131	158/180	51	32:19	3.35	15	11:4	3.40
145	174/188	40	12:28	6.58	18	5:13	3.68
147	176/188	27	3:24	18.59*	51	23:28	0.49
158	164/188	44	16:28	3.31	17	6:11	1.49
161	180/202	66	13:53	26.00*	11	11:0	NE
Gam11							
8	162/172	76	57:19	19.88*	3	2:1	NE
9	172/182	70	44:26	4.68	9	6:3	NE
16	220/228	67	30:37	0.73	13	7:6	0.08
24	220/266	74	13:61	33.80*	5	5:0	NE
28	220/228	78	31:47	3.30	1	1:0	NE
35	186/196	59	22:37	3.86	20	16:4	7.71
38	182/190	78	34:44	1.28	2	2:0	NE
39	220/228	76	27:49	6.46	1	1:0	NE

n_1 and n_2, sample size; G_1 and G_2, maximum likelihood G statistics for the hypotheses $n_{ij} = n_{ii} + n_{jj}$ and $n_{ik} : n_{jk}$, respectively. *Significant after a Bonferroni's correction for $\alpha = 0.05$ ($\chi^2 = 12.83$); NE, not estimated.

Table 2. Mendelian inheritance tests of three microsatellite loci of *Genipa americana*.

Seed-tree	Genotype	n_1	$n_{ij} : n_{ii} + n_{jj}$	G_1 (GL = 1)	n_2	$n_{ik} : n_{jk}$	G_2 (GL = 1)
Gam06							
9	142/144	33	8:25	9.19	46	1:45	54.13*
16	154/158	44	31:13	7.58	36	13:23	2.81
24	142/144	46	37:9	18.29*	31	0:31	NE
25	144/152	25	16:9	1.99	48	7:41	26.66*
26	144/152	65	55:10	34.30*	13	13:0	NE
28	142/144	27	4:23	14.77*	52	40:12	15.91*
29	142/144	66	32:34	0.06	14	0:14	NE
35	144/154	45	26:19	1.09	35	0:35	NE
37	144/172	50	6:44	32.62*	24	15:9	1.52
38	142/144	74	47:27	5.47	6	5:1	NE
79	142/144	14	7:7	0.00	57	0:57	NE
85	152/158	80	16:64	30.84*	0	0:0	NE
129	142/144	46	24:22	0.09	21	0:21	NE
131	144/152	39	19:20	0.03	26	24:2	21.94*
145	144/152	40	16:24	1.61	8	3:5	NE
147	142/144	53	6:47	36.04*	7	0:7	NE
Gam24							
1	140/160	67	39:28	1.81	13	12:1	10.97
9	140/148	43	1:42	50.11*	36	19:17	0.11
24	140/148	55	34:21	3.10	24	2:22	19.50*
35	140/148	41	8:33	16.36*	38	28:10	8.88
38	140/160	57	15:42	13.31*	22	21:1	22.36*
39	140/160	54	4:50	46.34*	25	22:3	16.31*
79	140/152	60	16:44	13.59*	20	12:8	0.81
85	134/148	23	8:15	2.16	57	54:3	55.51*
128	140/152	68	10:58	37.48*	11	9:2	4.82
131	148/152	47	38:9	19.25*	21	10:11	0.05
145	140/160	54	24:30	0.67	8	6:2	NE
147	140/160	74	33:41	0.87	5	4:1	NE
Gam41							
8	198/208	77	31:46	2.94	3	3:0	NE
9	198/208	38	14:24	2.66	41	29:12	7.27
14	198/208	35	20:15	0.72	45	19:26	1.09
16	198/208	35	20:15	0.72	45	19:26	1.09
21	166/174	29	13:16	0.31	1	0:1	NE
24	198/208	78	42:36	0.46	1	0:1	NE
25	198/208	65	27:38	1.87	4	3:1	NE
26	198/208	76	32:44	1.90	4	2:2	NE
28	198/208	48	19:29	2.10	32	30:2	29.40*
29	198/208	72	13:59	31.81*	8	5:3	NE
37	198/208	63	29:34	0.40	16	10:6	1.01
38	198/216	53	14:39	12.27*	27	19:8	4.61
39	198/208	66	22:44	7.48	12	6:6	NE
107	158/166	42	7:35	20.38*	31	12:19	1.59
129	198/208	75	28:47	4.87	1	0:1	NE
147	198/208	71	48:23	8.99	5	0:5	NE
158	198/208	52	14:38	11.51	9	6:3	NE

n_1 and n_2, sample size; G_1 and G_2, maximum likelihood G statistics for the hypotheses $n_{ij} = n_{ii} + n_{jj}$ and $n_{ik} : n_{jk}$, respectively. *Significant after a Bonferroni's correction for $\alpha = 0.05$ ($\chi^2 = 12.83$); NE, not estimated.

Table 3. Maximum likelihood G-test for the hypothesis of independent segregation between pairwise loci (1:1:1:1) of *Genipa americana*.

Seed-tree	G	Seed-tree	G	Seed-tree	G	Seed-tree	G
Gam01xGam02		Gam01xGam24		Gam02xGam24		Gam06xGam41	
8	84.61*	24	26.53*	9	11.90	9	29.81*
14	47.96*	35	87.54*	24	28.81*	16	0.54
21	13.48	38	154.45*	39	53.85*	24	28.00*
24	25.76*	39	48.11*	79	29.28*	25	16.05
25	24.19*	79	20.66*	128	76.20*	26	6.08
26	44.72*	85	63.31*	131	1.15	28	36.85*
37	71.75*	128	57.80*	147	19.01	29	12.46
39	12.03	Gam01xGam41		Gam02xGam41		37	15.04
79	14.02	8	84.70*	8	34.98*	38	16.44
107	2.88	14	59.53*	9	13.41	129	32.80*
128	16.59	21	12.80	14	2.33	147	21.20*
129	8.89	24	21.98*	21	2.74	158	14.48
145	15.76	25	18.04	24	12.22	Gam11xGam24	
147	12.58	26	49.09*	25	8.71	9	5.00
158	9.86	28	7.85	26	7.24	24	95.72*
161	78.47*	37	42.71*	29	7.64	35	38.08*
Gam01xGam06		38	120.61*	37	34.70*	38	81.14*
24	19.82*	39	3.70	39	3.94	39	64.63*
25	23.49*	107	5.35	107	8.44	Gam11xGam41	
26	65.98*	129	4.87	129	20.57*	8	19.45
28	15.89	147	21.85*	147	7.88	9	2.62
35	113.74*	158	4.53	158	8.24	16	8.21
37	37.48*	Gam02xGam06		Gam06xGam11		24	85.30*
38	166.65*	9	67.61*	9	38.62*	28	17.49
85	10.78	24	10.50	16	13.89	38	19.67*
129	27.50*	25	26.98*	24	83.93*	39	14.99
145	13.18	26	13.20	28	42.94*	Gam24xGam41	
147	32.76*	29	6.42	35	41.62*	9	4.69
158	16.56	37	20.19*	38	26.17*	24	39.56*
Gam01xGam11		129	33.69*	Gam06xGam24		38	51.59*
8	81.03*	131	18.91	9	79.44*	39	46.12*
24	75.58*	145	19.21	24	39.02*	147	25.51*
28	15.76	147	10.04	35	29.49*		
35	90.73*	158	9.28	38	67.40*		
39	18.31	Gam02xGam11		85	74.62*		
Gam01xGam24		8	13.79	131	18.78		
39	48.11*	9	10.93	147	20.91*		
79	20.66*	24	53.78*				
128	57.80*	39	22.46*				

*Significant after a Bonferroni's correction for $\alpha = 0.05$, 0.00037 ($\chi^2 = 19.61$); G, G-test with three degrees of freedom.

Table 4. Genotypic disequilibrium between pairwise microsatellite loci in adult trees and regenerants of *Genipa americana*.

Pairwise loci	Adult trees	Regenerants
Gam01xGam02	0.10000	0.56875
Gam01xGam06	0.09018	0.69554
Gam01xGam11	0.10446	0.00893
Gam01xGam24	0.22857	0.00804
Gam01xGam41	0.21875	0.05893
Gam02xGam06	0.28571	0.00179
Gam02xGam11	0.86607	0.06607
Gam02xGam24	0.58304	0.32143
Gam02xGam41	0.72232	0.64018
Gam06xGam11	0.46696	0.48214
Gam06xGam24	0.74018	0.08036
Gam06xGam41	0.11161	0.00268
Gam11xGam24	0.58571	0.11607
Gam11xGam41	0.58571	0.00179
Gam24xGam41	0.47232	0.15982

The values represent the probability of genotypic disequilibrium after 1200 permutations of alleles among individuals; after Bonferroni's corrections, $P = 0.00089$ ($\alpha = 0.05$).

DISCUSSION

Overall, the results show that all six loci segregated according to the Mendelian rules of 1:1. Deviations were detected in some progenies, but these were not repeated in all of the progenies at the same locus, indicating that the observed deviations could be attributed to sample drift, considering that the fruits contained many seeds (>200) and were sampled at the maximum of 80 seeds per seed-tree. Moreover, deviations occurred in only three cases simultaneously for both realized tests ($n_{ij} : n_{ii} + n_{jj}$ and $n_{ik} : n_{jk}$), suggesting that the observed deviations were random and that these loci segregate according to Mendelian rules. Therefore, the six molecular markers can be considered genetic markers.

The linkage observed between pairs of loci for several progenies may be a true genetic linkage, or may originate from individual locus deviations from a 1:1 Mendelian segregation. Loci with deviation from a 1:1 segregation may exhibit linkage disequilibrium. Some progenies showed deviations in some loci, and were used for the linkage tests. Therefore, segregation deviations in an individual locus may have generated the significant G-test values. For example, in 43 of the 74 linkage tests (58%), significant linkage was present in at least in one locus, with significant deviation from a 1:1 segregation. However, for the pairwise loci Gam01xGam24, there was strong evidence of linkage, because all of the progeny exhibited significant linkage. Ideally, one of these loci should be excluded for some population genetic analyses, particularly mating system and parentage analysis studies.

Furthermore, some linkage may have occurred due to small sample sizes. Tambarussi et al. (2013) studied two populations of *Cariniana legalis*, and detected deviations from the null hypothesis of a 1:1:1:1 segregation between pairwise loci; the authors attribute their results to the low number of descendants used in the analysis. This may have occurred in the present study for some specific cases, because although a substantial number of descendants of each seed-tree was used (up to 80 seeds), only the seeds with both alleles of maternal origin in the two loci were included in the tests, which substantially reduced the number of descendants in the analysis. A similar result was reported by Tarazi et al. (2010), who studied 20 samples per progeny collected from 28 *Copaifera langsdorffii* seed-trees. Furthermore, the observed deviations from the null hypothesis for the segregation of individual loci, and for the pairwise loci, may have been indirectly caused by prezygotic effects, such as meiotic drift, differential gametic viability, differential reproductive success (gametic incompatibility); or by postzygotic mechanisms, such as inbreeding depression due to natural selection (Hufford and Hamrick, 2003), particularly because seedlings were analyzed, which represent a post-fertilization stage. Pre- and postzygotic effects were suggested by Tarazi et al. (2010) as the causes of the deviations found in their study on *C. langsdorffii*.

Genotypic disequilibrium in molecular markers is one of the basic assumptions for their use in studies of genetic diversity and structure, mating systems, and parentage analyses. We found no evidence of genotypic disequilibrium between pairs of loci in adults and regenerants, suggesting linkage equilibrium. This result is also an indication that the observed deviations in the linkage tests may have been caused by sample drift.

The six loci exhibited Mendelian inheritance, genotypic equilibrium, and were not linked, with the exception of the pairwise loci Gam01xGam24. Therefore, we suggest excluding Gam01 and Gam24 from population genetic analyses, in particular from mating system and parentage analysis studies.

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