Short Communication

Mendelian inheritance, linkage, and genotypic disequilibrium in microsatellite loci of *Hymenaea stigonocarpa* Mart. ex Hayne (Fabaceae-Caesalpinioideae)

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ABSTRACT. *Hymenaea stigonocarpa* is a deciduous and monoecious Neotropical tree species pollinated by bats. Due to overexploitation and habitat destruction, the population size has drastically diminished in nature. No previous study has investigated Mendelian inheritance, linkage, and genotypic disequilibrium in the available microsatellite markers in this species. So, our aim was to estimate these parameters using six microsatellite loci in a sample of 470 adults and 219 juveniles

from two populations of *H. stigonocarpa*. In addition, 30 seeds per tree from 35 seed-trees were collected. Each seed was kept record of the seed-trees and fruit origin. Based on the six microsatellite loci, we found that only 10.6% of the cases showed significant deviations from Mendelian segregation and 15.3% showed linkage. We detected no evidence of genotypic disequilibrium between the loci in the adult trees or juveniles. Thus, our results suggest that these loci can be used with great accuracy in future genetic analyses of *H. stigonocarpa* populations.

Key words: Forest fragment; Jatobá; Microsatellite; Neotropical; Open-pollinated seeds

INTRODUCTION

Hymenaea stigonocarpa Mart. ex Hayne belongs to the family Fabaceae-Caesalpinioideae, and is widely distributed in the Brazilian savannah. The species is deciduous, monoecious, and pollinated by bats. Studies involving controlled mating of *H. stigonocarpa* indicate that the species is self-compatible (Gibbs et al., 1999). Estimates of outcrossing rate, based on genetic markers, have confirmed these results, indicating that the species has a mixed mating system (Moraes and Sebbenn, 2011). The economic importance of *H. stigonocarpa* is associated with the use of the wood for naval and civil construction (Lorenzi, 1992; Botelho et al., 2000). The species has been intensively exploited in the past by the naval industry, due to the excellent quality of its wood in terms of durability and resistance to rotting. Because of this, and owing to the widespread destruction of its habitat in the savannah, the species is now found only in small remnant populations or as isolated trees in fields and pastures.

Studies of the genetic consequences of this reduction of the natural population size have been made possible due to the identification of a group of polymorphic microsatellite loci (Ciampi et al., 2008). However, to our knowledge, no study on Mendelian inheritance, linkage, and genotypic disequilibrium has been conducted for the developed microsatellite loci. It is important to confirm whether the marker loci are genetic loci. In this study, our goal was to estimate the Mendelian inheritance, linkage, and genotypic disequilibrium in six microsatellite loci transferred to *H. stigonocarpa*. This knowledge will infer greater reliability in the use these markers for future population genetic studies.

MATERIAL AND METHODS

The study was conducted in an area of 23 x 32 km (736 km^2), using isolated trees and trees occurring in a forest fragment near the city of Inocência (20°07'S, 51°44'W; 373 m above sea level), in the State of Mato Grosso do Sul, Brazil. The climate conditions are tropical with a dry winter and a wet summer. The average rainfall is 1232.2 mm, with an average annual temperature of 24.5°C. The regional biome is characterized by savannah with high levels of anthropogenic disturbance.

The collection of open-pollinated seeds was made in two populations of *H. stigonocarpa.* One population was located in a pasture area where trees are isolated or in small clusters (pasture) where 359 adult trees were leaf sampled and genotyped. The second

population was located in a forest fragment (forest) in which 111 adult trees and 219 juveniles were sampled and genotyped. Additionally, we collected and genotyped open-pollinated seeds from 20 seed trees in the PPA and 15 seed trees in PFF, with 30 seeds per tree in different fruits, adding up to a total of 1050 seeds. Each seed was kept record of the seed trees and fruit origin.

The DNA extraction from leaves of seed-trees and germinated seeds was carried out using the method of Doyle and Doyle (1987). DNA quantification of the samples was made using an electrophotometer. The amplification reactions were performed according to the method presented in Ciampi et al. (2008). The amplified DNA fragments were separated on a Fragment Analyzer™ Automated CE System (Advanced Analytical Technologies, Ames, IA, USA) using the 35-500 bp dsDNA Reagent Kit (Advanced Analytical Technologies). Reading of the alleles was done using the PROSize™ v. 2.0 software [Inc. (AATI), Ames, IA, USA]. Initially, nine di-nucleotide microsatellite loci, developed by Ciampi et al. (2008) for *Hymenaea courbaril* L. were tested. Of these nine loci, six (HC14, HC17, HC33, HC35, HC40, and HC49) were successfully transferred to *H. stigonocarpa*, which were subsequently used in the present study.

The Gillet and Hattemer (1989) method was used to estimate Mendelian inheritance. The model assumes that loci with regular segregation meet three basic requirements: i) regular meiotic segregation during ovule production, ii) random fertilization within the ovule by any type of pollen, and iii) no selection for viability between the analyzed seeds. As the model assumes co-dominance among all the alleles, two further conditions must be met: the first is that all the progeny of a tree must have a maternal allele. The second condition is that, in cases of heterozygous parent trees (e.g., A_iA_j, $i \neq j$), the following must occur: a) each progeny must have an allele of the maternal tree, A_i or A_j; b) the number of heterozygous progeny A_iA_j (n_{ij}) must equal the sum of the 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 equal the number of heterozygous progeny A_jA_k (n_{jk}), or $n_{ik} = n_{jk}$, where k ≠ i, j. To test if there were significant differences between the observed and expected values of segregation, we used the *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 $E(n) = 0.5(n_{ij} + n_{ii} + n_{jj})$.

We also analyzed if the microsatellite loci were linked. The linkage test was done only in the progeny of heterozygous seed-trees. In this case, the null hypothesis is a regular Mendelian segregation of 1:1:1:1. This hypothesis was accepted or discarded based on a maximum likelihood *G*-test (Sokal and Rohlf, 1981):

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

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, *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 follows:

$$E(n) = 0.25(n_{ik} + n_{il} + n_{jk} + n_{jl}). \qquad \text{Equation 3}$$

The genotypic disequilibrium test was performed in juveniles and adult trees between all pairwise loci using the FSTAT program version 2.9.3.2 (Goudet, 2002). Bonferroni's correction at 95% probability ($\alpha = 0.05$) was applied in all analyses to avoid false positives.

RESULTS

After Bonferroni's correction, we found that the microsatellite loci did exhibit Mendelian inheritance, although there were few significant deviations from the expected segregation pattern in 12 of 113 tests (10.6%; Table 1). We also observed significant locus linkage in 24 of 156 linkage tests performed (15.3%; Table 2), suggesting that some pairs of loci were physically linked. However, these occurrences of linkage appeared in different pairs of loci in the two populations sampled, suggesting they are not genetically linked. The genotypic disequilibrium was tested in samples of adult trees and juveniles from the forest and in adult trees only in the pasture. We found no significant results after Bonferroni's correction, suggesting that the loci were all in linkage equilibrium (Table 3).

DISCUSSION

In general, we found that all microsatellite loci exhibited a Mendelian segregation of 1:1. A few cases of deviations were detected in some progenies, but these never occurred within all progenies at the same locus. This suggests that the six investigated loci segregate according to the expected ratios. Studies of microsatellite loci in other species have also detected low segregation deviations. Carneiro et al. (2012) observed deviations from the expected 1:1 segregation only in locus HC33 (the same locus as used in the present study) in *H. courbaril*. Tambarussi et al. (2013) observed deviations from the expected 1:1 segregation in 3.7% of the tests in *Cariniana legalis*, and Manoel et al. (2015) observed deviations from the expected segregation of 1:1 in 29% of tests in *Genipa americana*.

Our results also indicated that the loci were not linked and that they were in genotypic equilibrium. Genetic linkage is caused by an absence of low recombination rate of loci that are close on the same chromosome, which keeps alleles together when inherited (Hartl and Clark, 2010). However, in this study, the majority of the microsatellite pairs revealed an absence of linkage. The HC14 locus stands out for showing significant linkage in 14 of 54 cases (25.9%). These linkages between pairs of loci found in several progenies may be a true genetic linkage, or it may be caused by deviations from a 1:1 Mendelian segregation (Manoel et al., 2015). We propose that it is the latter because 11 of the 14 significant linkage tests involving locus HC14 were detected in families that also showed significant segregation deviation. Another reason for the observed linkage could be the sample size used. Tarazi et al. (2010) studied *Copaifera langsdorffii* and concluded that using a reduced number of seedlings per family in combination with a high number of alleles per locus, deviation was expected. Tarazi et al. (2010) therefore recommend future studies to, if possible, adjust the seedling sample size to the species mean number of alleles, in order to achieve reliable frequency estimates. Similar results were reported by Carneiro et al. (2012), who analyzed 13 to 20 seeds per family collected from *H. courbaril* and Tambarussi et al. (2013), who studied two populations of *C. legalis* with samples of 40 to 100 seeds.

Table 1. Mendelian inheritance tests for six microsatellite loci in *Hymenaea stigonocarpa*.

Seed-tree	Genotype	N	N_1	$n_{ij} : n_{ii} + n_{jj}$	*G*	Seed-tree	Genotype	N	N_1	$n_{ij} : n_{ii} + n_{jj}$	*G*
HC14						*HC17*					
24	126130	19	16	3:13	0.43	37	128132	30	19	7:12	1.33
37	130136	30	26	1:25	27.57*	240	110114	24	21	1:20	21.07*
240	128138	24	18	5:13	3.68	256	120130	24	16	3:13	6.74
358	126136	30	26	11:15	0.62	292	120130	20	15	2:13	9.01
610	128136	18	15	3:12	5.78	313	116120	28	20	3:17	10.82
702	126130	29	28	1:27	30.19*	339	128134	19	16	8:8	0.0
703	128132	29	22	4:18	9.64	342	120128	28	18	8:10	0.22
707	126130	30	29	4:25	16.93	358	126130	30	19	9:10	0.05
712	128132	30	23	1:22	23.66*	368	136140	24	16	7:9	0.25
714	126130	25	21	1:20	21.07*	700	116120	30	22	6:16	4.72
715	122130	30	27	2:25	23.17*	702	130136	29	22	5:17	6.92
724	128132	24	19	3:16	9.77	703	116120	29	20	4:16	7.71
HC33						707	116120	30	22	7:15	2.98
15	102112	30	27	6:21	8.83	710	126136	29	19	7:12	1.33
37	110116	30	27	11:16	0.93	715	114124	30	23	10:13	0.39
368	98110	22	17	8:9	0.06	716	116124	30	23	12:11	0.04
703	112116	29	29	3:26	20.91*	721	116122	30	28	8:20	5.31
715	112116	30	30	15:15	0.0	723	116122	25	20	10:10	0.0
716	112116	30	30	6:24	11.57	724	120128	24	24	3:21	15.19
721	112116	30	29	8:21	6.04	*HC35*					
722	108112	30	30	5:25	14.56	11	280290	27	27	3:24	18.59
723	108112	25	25	7:18	5.01	15	270280	30	30	2:28	26.89*
						24	290296	19	18	3:15	8.73
						34	280290	18	15	4:11	3.40
						37	284296	40	23	5:18	7.80
						240	286292	24	21	5:16	6.06
						249	290296	29	20	16:4	7.71
						256	290296	24	17	11:6	1.49
						272	280290	23	15	5:10	1.70
						313	274286	28	25	9:16	1.99
						334	280290	21	19	3:16	9.77
						339	270280	19	18	6:12	2.04
						342	280290	28	27	6:21	8.83
						358	280290	30	30	2:28	26.89*
						368	280290	22	21	3:18	11.89
						610	286290	18	15	3:12	5.78
						700	270280	30	23	6:17	5.48
						702	280290	29	23	7:16	3.62
						704	280290	30	26	6:20	7.95
						707	280290	30	22	7:15	2.98
						712	276290	30	22	10:12	0.18
						715	280290	30	27	16:11	0.93
						721	280288	30	29	4:25	16.93
						722	278288	30	22	2:20	17.09
						723	286296	25	15	2:13	9.01
						724	276286	26	23	2:21	18.30
HC40						*HC49*					
11	172180	27	24	14:10	0.67	15	106110	30	30	9:21	4.94
15	172178	30	29	19:10	2.84	24	104108	19	16	5:11	2.31
37	174180	30	29	8:21	6.04	37	104108	30	25	15:10	1.01
240	174180	24	18	6:12	2.04	240	104108	24	18	8:10	0.22
249	172178	29	26	15:11	0.62	272	106112	23	22	5:17	6.92
321	176180	19	15	1:14	13.45	292	102106	20	19	6:13	2.64
334	170180	21	21	8:13	1.20	299	100106	16	15	6:9	0.60
339	170178	19	19	8:11	0.48	313	100106	28	28	11:17	1.30
342	174180	28	21	13:8	1.20	321	98102	19	19	8:11	0.48

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Table 1. Continued.

Seed-tree	Genotype	N	N_1	$n_{ij} : n_{ii} + n_{jj}$	*G*	Seed-tree	Genotype	N	N_1	$n_{ij} : n_{ii} + n_{jj}$	*G*
HC40						*HC49*					
358	170176	30	23	6:17	5.48	334	98102	21	20	11:9	0.20
610	162172	18	15	9:6	0.60	339	98104	19	18	6:12	2.04
702	174180	29	20	5:15	5.23	342	98102	28	15	8:7	0.07
703	174180	29	20	7:13	1.83	358	102108	30	26	19:7	5.75
704	174180	30	27	2:25	23.17*	368	104110	22	20	15:5	5.23
707	172180	30	25	10:15	1.01	610	106112	18	17	11:6	1.49
710	172180	29	29	6:23	10.63	700	100106	30	20	13:7	1.83
712	174180	30	28	10:18	2.32	702	100106	27	22	11:11	0.00
714	176182	25	22	6:16	4.72	704	106112	30	29	27:2	25.65*
715	174182	30	30	18:12	1.21	707	106112	30	27	20:7	6.53
716	174180	30	30	13:17	0.53	710	106112	29	28	20:8	5.31
721	174180	30	29	19:10	2.84	712	104110	30	23	12:11	0.04
722	172180	30	23	10:13	0.39	715	102108	30	16	7:9	0.25
723	168178	25	23	9:14	1.10	716	104108	30	24	7:17	4.30
						721	106112	30	28	16:12	0.57

N and N_1 = sample size and sample size used for *G* test; *G* = maximum likelihood *G* statistics for the hypothesis of $n_{ij} : n_{ii} + n_{jj}$. *Significant after Bonferroni's correction for $\alpha = 0.05$: P = 0.00023 ($\chi^2 = 20.13$)..

Table 2. Maximum likelihood *G*-test for the hypothesis of independent segregation between pairwise loci (1:1:1:1) of *Hymenaea stigonocarpa*.

Seed-tree	*G*	Seed-tree	*G*	Seed-tree	*G*	Seed-tree	*G*
HC14 x HC17		*HC14 x HC35*		*HC14 x HC49*		*HC17 x H35*	
37	37.45*	342	2.72	334	9.62	610	11.77
358	8.11	358	11.67	339	4.35	700	3.36
368	10.45	610	9.96	342	1.95	702	6.38
700	24.72*	702	5.26	358	9.38	707	0.92
702	7.00	707	0.07	610	13.07	715	13.94
703	4.19	712	9.78	702	4.03	721	25.57*
707	0.07	715	29.01*	707	0.90	723	13.70
714	6.87	724	3.72	715	22.75*	724	4.06
715	25.22*	*HC14 x HC40*		*HC17 x HC33*		*HC17 x HC40*	
724	1.43	240	18.11	37	10.35	37	4.57
715	5.33	334	10.27	368	15.84	240	18.04
716	11.29	339	6.85	703	0.98	339	5.12
721	3.77	342	2.75	715	8.78	342	3.17
722	15.40	358	9.48	716	9.42	358	6.72
723	14.46	610	7.54	721	4.71	610	11.71
HC14 x HC33		702	19.67*	723	8.71	702	22.13*
37	46.61*	703	4.45	*HC17 x H35*		703	3.29
703	5.18	707	7.52	37	6.34	707	12.21
715	27.35*	712	20.01	240	12.87	710	7.37
HC14 x HC35		715	29.24*	256	22.31*	714	20.43
37	33.79*	*HC14 x HC49*		313	5.12	715	8.97
240	13.04	24	9.68	339	3.56	716	0.68
272	24.56*	37	44.00*	342	1.92	721	1.98
334	6.59	240	9.37	358	12.34	723	2.17
339	7.19	272	27.19*	368	14.71		
HC17 x HC49		*HC33 x HC40*		*HC35 x HC40*			
37	2.03	15	22.21*	342	4.19		
240	26.52*	37	14.36	358	11.15		
292	3.82	703	3.13	610	5.49		
313	3.71	715	5.33	702	18.59		
321	4.57	716	11.29	704	32.29*		
339	1.13	721	3.77	707	9.33		
342	2.54	722	15.40	712	9.23		
358	6.37	723	14.46	715	11.46		
240	26.52*	37	14.36	358	11.15		
292	3.82	703	3.13	610	5.49		
313	3.71	715	5.33	702	18.59		
321	4.57	716	11.29	704	32.29*		
339	1.13	721	3.77	707	9.33		
368	9.80	*HC33 x HC49*		721	25.08*		
610	13.55	15	14.63	722	7.19		
700	2.99	37	14.27	723	2.08		
702	9.02	368	7.28	*HC35 x HC49*			
707	1.40	715	5.76	15	10.82		
710	9.04	716	9.09	24	14.73		
715	8.89	721	10.16	37	7.16		
716	2.88	723	11.98	*HC40 x HC49*			
721	4.31	*HC35 x HC40*		710	2.02		
368	9.80	*HC33 x HC49*		721	25.08*		
610	13.55	15	14.63	722	7.19		
HC33 x HC35		11	13.62	715	2.89		
15	23.31*	15	12.08	716	3.31		
37	13.51	37	5.09	721	4.16		
715	5.95	240	4.92	723	0.80		
721	15.41	249	0.55				
722	14.48	334	6.26				
723	20.22	339	6.02				

*Significance after Bonferroni's correction for $\alpha = 0.05$: P = 0.00037 ($\chi^2 = 20.66$). *G* = *G*-test for d.f. = 3.

Table 3. Genotypic disequilibrium between pairwise microsatellite loci in juveniles and adult trees of *Hymenaea stigonocarpa*.

Pairwise loci	Forest: adults	Forest: juveniles	Pasture: adults
HC33 x HC35	0.07533	0.32200	0.47600
HC33 x HC40	0.11133	0.05533	0.31333
HC33 x HC49	0.71867	0.05733	0.38800
HC33 x HC14	0.19200	0.01600	0.43067
HC33 x HC17	0.03200	0.05533	0.05200
HC35 x HC40	0.02000	0.98533	0.68333
HC35 x HC49	0.75133	0.04867	0.04133
HC35 x HC14	0.33600	0.03800	0.42467
HC35 x HC17	0.58000	0.06467	0.24667
HC40 x HC49	0.83600	0.55533	0.31533
HC40 x HC14	0.45333	0.70000	0.01533
HC40 x HC17	0.45267	0.11200	0.05000
HC49 x HC14	0.54000	0.80200	0.43867
HC49 x HC17	0.75067	0.06667	0.06200
HC14 x HC17	0.87400	0.48133	0.44667

The values represent the probability of genotypic linkage after 1500 permutations of alleles among individuals. Probability after Bonferroni's correction: $P = 0.00067$ ($\alpha = 0.05$).

Moraes et al. (2007) observed genotypic disequilibrium in adult *H. stigonocarpa* between the HC33 and HC49 loci. The population they studied was located near the populations studied in this research, indicating that linkage disequilibrium can occur in closely located places. In the present study, we observed no linkage disequilibrium that could have broken family structure. This may be explained by natural thinning and indirect selection during plant development of the loci analyzed.

It has been suggested that prior knowledge of independent segregation of alleles at different loci is one of the key items for the study of the mating system of plant species (Ritland and Jain, 1981). The results presented in this study show that even with the transfer of microsatellite loci to *H. stigonocarpa*, these loci could be characterized as molecular genetic markers that may be used in future studies of the genetic behavior of these two natural populations.

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