



Brief Note

Mendelian inheritance, linkage and genotypic disequilibrium in microsatellite loci isolated from *Hymenaea courbaril* (Leguminosae)

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ABSTRACT. The Neotropical tree *Hymenaea courbaril*, locally known as Jatobá, is a valuable source of lumber and also produces comestible and medicinal fruit. We characterized Mendelian inheritance, linkage and genotypic disequilibrium at nine microsatellite

loci isolated from *H. courbaril*, in order to determine if they would provide accurate estimates of population genetic parameters of this important Amazon species. The study was made on 250 open-pollinated offspring originated from 14 seed trees. Only one of nine loci presented significant deviation from the expected Mendelian segregation (1:1). Genotypic disequilibrium between pairwise loci was investigated based on samples from 55 adult and 56 juvenile trees. No genetic linkage between any paired loci was observed. After Bonferroni's corrections for multiple tests, we found no evidence of genotypic disequilibrium between pairs of loci. We conclude that this set of loci can be used for genetic diversity/structure, mating system, gene flow, and parentage analyses in *H. courbaril* populations.

Key words: Brazilian Amazon; *Hymenaea courbaril*; SSR loci; Mendelian inheritance; Neotropical tree; Population genetics

INTRODUCTION

Hymenaea courbaril L. (Leguminosae) is one of the most important timber species currently logged in the Brazilian Amazon. In general this large tree occurs in very low population densities in natural stands (<1 tree/ha). Owing to its intense exploitation in the Amazon, populations of *H. courbaril* have been significantly reduced (Lacerda et al., 2008a). Investigations about the impacts of forest logging on the genetic diversity, mating system and gene flow using genetic markers, are very important for the development of species' appropriate sustainable logging practices. For *H. courbaril*, such studies have recently started (Dunphy et al., 2004; Lacerda et al., 2008a,b; Sebbenn et al., 2008) after the development and characterization of a set of polymorphic microsatellite loci for the species (Ciampi et al., 2000). However, to ensure the appropriate use of these loci for genetic analyses of *H. courbaril* populations, it is important to verify if the microsatellite loci display Mendelian inheritance and if they are associated.

Here, we characterized the Mendelian inheritance, linkage and genotypic disequilibrium in nine specific microsatellite loci previously developed for *H. courbaril*, an important timber species in the Brazilian Amazon, for robust application in population genetic studies such as genetic diversity and structure, mating system, gene flow, and parentage analyses in this species.

METHODS AND RESULTS

Mendelian inheritance and linkage disequilibrium between loci were examined based on 250 open-pollinated offspring collected from 14 seed trees (13 to 20 seeds per seed tree) in a 546-ha plot in the Tapajós National Forest, Pará State, Brazil. We also sampled 55 adult and 56 juvenile trees to study the genotypic disequilibrium between pairwise

loci. For DNA extraction, two cambium samples were collected per adult and/or juvenile trees. The cambium samples were stored in microtubes (1.5 mL) containing a DNA extraction buffer solution [CTAB buffer (1/3) and ethanol (2/3)], and stored at -20°C until DNA extraction. From nursed saplings (ca. 45 cm high), two leaflets were collected, dried on silica gel, and maintained at -20°C until DNA extraction. Total genomic DNA was extracted following a standard CTAB protocol (Doyle and Doyle, 1987). Nine nuclear microsatellite markers previously developed for *H. coubaril* (Ciampi et al., 2000) were fluorescently labeled for multiplexing analysis as described by Carneiro et al. (2011). The *in vitro* amplification of the microsatellite loci was carried out in a total volume of 10 μL containing 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl_2), 200 μM dNTPs, 2.5 mg/mL bovine serum albumin, 1.25 μM of each forward and reverse primer, 1 U Taq DNA polymerase and 5.0 ng genomic DNA using a Veriti thermal cycler (Life Technologies). The PCR conditions consisted of an initial heating at 95°C for 15 min followed by 30 cycles of 94°C for 1 min, annealing at the melting temperature of each primer pair for 1 min, and 72°C for 1 min, and a final extension at 72°C for 30 min. The PCR products were visualized on 1.5% agarose gels stained with ethidium bromide under UV light and sized using 1 kB Plus DNA Ladder (Gibco, USA). After dilution the PCR products were sized using GeneScan ROX 500 sizing standard in a 3130XL platform (Life Technologies). The amplified fragments (alleles) were analyzed using GeneMapper v. 1.0 (Life Technologies).

Mendelian inheritance of the microsatellite loci was determined according to Gillet and Hattermer (1989). The genetic linkage between loci was tested against the hypothesis that maternal seed trees, considering heterozygous at two loci ($A_iA_jB_iB_j$) and in the absence of linkage, would result in equal proportions (test of 1:1:1:1) of the different maternal gametic types (A_iB_i , A_iB_j , A_jB_i , and A_jB_j). Thus, the observed genotypes of maternal families from seed trees heterozygous at two loci were compared to the expected gametic proportion based on no linkage (1:1:1:1). This hypothesis was evaluated by a maximum likelihood G-test carried out for each seed tree. We also tested the genotypic disequilibrium between pairwise loci in adults and juveniles, using the FSTAT program (Goudet, 1995) and a Bonferroni's correction ($\alpha = 0.05$) to avoid false positives.

The results showed a significant deviation from the expected 1:1 Mendelian segregation in only one family in the locus *Hc33* (Table 1). However, when we grouped the families with the same maternal genotype and re-analyzed the segregation, there was no observed deviation from the expected 1:1 segregation. After Bonferroni's correction only eight (0.7%) of 106 linkage tests performed (Table 2) were significant, suggesting linkage between some pairs of loci. In all cases in which significant linkage was observed, it occurred in different pairs of loci of different samples and in only one family. On the other hand, the majority of families adhered to the expected 1:1:1:1 Mendelian inheritance for the same pairs of loci analyzed. Hence, we conclude that the nine microsatellite loci analyzed are not linked. A small number of pairwise loci showed linkage, which probably can be an effect of the small sampling size within the families. After Bonferroni's correction, the results showed no significant evidence of genotypic disequilibrium between pairwise loci (Table 3).

Table 1. Mendelian inheritance tests for nine microsatellite loci in *Hymenaea courbaril*.

Locus-seed tree	Genotype	N	$n_{ij} : n_{ii} + n_{jj}$	$n_{ik} : n_{jk}$	G_1	G_2
Hc6						
400131	5864	17	4:13	0:0	5.02	-
400440	6468	19	3:6	1:9	1.02	7.36
500182	5864	15	8:7	0:0	0.07	-
700119	5864	19	11:8	0:0	0.48	-
702357	5864	10	4:6	0:0	0.40	-
Hc12						
401048	158160	20	8:12	0:0	0.81	-
401169	158160	17	13:4	0:0	5.02	-
501785	158160	14	8:6	0:0	0.29	-
602696	158160	20	13:7	0:0	1.83	-
700119	158160	20	12:8	0:0	0.81	-
702357	158160	13	6:7	0:0	0.08	-
Hc42						
400131	124140	20	2:6	12:0	2.09	-
400440	124126	19	2:5	6:6	1.33	-
401048	124140	18	1:1	10:6	-	1.01
501383	124144	14	1:3	3:7	1.05	1.65
606001	122124	12	5:7	0:0	0.33	-
Hc14						
400131	118122	20	4:16	0:0	7.71	-
501383	118122	16	4:12	0:0	4.19	-
502348	118122	12	4:8	0:0	1.36	-
700119	118122	20	12:8	0:0	0.81	-
Hc40						
400131	170174	20	1:5	7:7	2.91	-
400440	174182	20	1:2	5:12	0.34	2.97
401048	166188	20	0:1	9:10	-	0.05
401169	160184	17	0:0	6:11	-	1.49
500182	170180	17	2:2	7:6	-	0.08
501383	180188	13	1:2	3:7	0.34	1.65
501785	168172	13	2:2	6:3	-	1.02
600959	168188	17	20	4:11	-	3.40
602696	174184	18	0:0	12:6	-	2.04
700119	168184	14	1:2	6:5	0.34	0.09
Hc34						
400131	188190	19	2:5	1:11	1.33	9.75
400440	190192	18	2:1	5:10	0.34	1.70
401048	164190	10	4:6	0:0	0.40	-
602696	190192	16	5:3	7:1	0.51	5.06
700119	190192	16	3:6	2:5	1.02	1.33
702357	190192	12	0:0	8:4	-	1.36
Hc33						
501785	108110	13	5:8	0:0	0.70	-
606001	108110	18	2:16	0:0	12.40*	-
Grouped	108110	31	7:24	0:0	9.86	-
Hc17						
400131	106112	20	5:2	7:6	1.33	0.08
400440	110112	16	11:5	0:0	2.31	-
500182	106114	16	2:5	2:7	1.33	2.94
501383	106110	15	5:3	1:6	0.51	3.96
501785	106110	18	0:4	12:2	-	7.92
602696	106110	20	8:12	0:0	0.81	-
700119	110114	20	4:4	7:5	0.00	0.33
Hc25						
400131	120152	20	1:7	6:6	5.06	-
401048	122152	18	2:7	1:8	2.94	6.20
600959	122154	17	2:5	1:9	1.33	7.36
602696	120154	16	1:9	2:4	7.36	0.68
606001	122150	15	0:2	8:5	-	0.70

N = sample size; G_1 and G_2 = maximum likelihood G statistics for the hypothesis of $n_{ij} = n_{ii} + n_{jj}$ and $n_{ik} = n_{jk}$ respectively. Probability of Bonferroni's corrections $\alpha = 0.001$ ($\chi^2_{Table} = 10.83$).

Table 2. Maximum likelihood G test for testing the hypothesis of independent segregation between pairs of microsatellite loci (1:1:1:1) in *Hymenaea courbaril*.

Locus	Seed-tree	G	Locus	Seed-tree	G	Locus	Seed-tree	G	Locus	Seed-tree	G	Locus	Seed-tree	G
Hc6xHe12	700119	7.14	He12xHe42	401048	1.45	He14xHe40	400131	5.38	He40xHe34	401169	0.57	He42xHe14	501383	7.65
Hc6xHe12	702357	4.95	He12xHe42	401169	0.25	He14xHe40	502348	4.87	He40xHe34	400440	3.29	He42xHe14	400131	16.74*
Hc6xHe42	400440	3.27	He12xHe42	700119	0.78	He14xHe40	700119	0.39	He40xHe34	602696	2.16	He42xHe14	400440	2.45
Hc6xHe42	500182	4.03	He12xHe42	702357	0.68	He14xHe40	400131	16.80*	He40xHe34	700119	0.51	He42xHe14	401048	3.40
Hc6xHe14	700119	2.70	He12xHe14	700119	3.18	He14xHe34	502348	0.90	He40xHe34	400131	9.86	He42xHe40	606001	4.52
Hc6xHe14	702357	3.49	He12xHe14	401048	0.82	He14xHe34	700119	1.68	He40xHe34	401048	5.88	He42xHe40	501383	4.93
Hc6xHe40	500182	3.85	He12xHe40	401169	4.68	He14xHe17	400131	7.30	He40xHe34	500182	3.55	He42xHe34	501383	4.72
Hc6xHe40	700119	4.79	He12xHe40	501785	0.17	He14xHe17	502348	8.54	He40xHe34	600959	5.76	He42xHe34	400440	7.76
Hc6xHe40	400131	10.62	He12xHe40	602696	3.80	He14xHe17	700119	3.67	He40xHe33	501785	3.51	He42xHe34	401048	11.23
Hc6xHe40	400440	6.07	He12xHe40	700119	0.78	He14xHe25	400131	8.26	He40xHe17	400131	0.78	He42xHe34	400131	17.32*
Hc6xHe40	500182	2.05	He12xHe40	702357	7.22	He14xHe25	502348	6.59	He40xHe17	400440	10.71	He42xHe33	606001	12.69*
Hc6xHe40	700119	3.75	He12xHe34	700119	1.65	He14xHe25	700119	15.76*	He40xHe17	401048	2.97	He42xHe17	501383	3.96
Hc6xHe34	702357	8.37	He12xHe34	401048	5.38	He40xHe17	401169	3.68	He42xHe17	400440	4.82	He17xHe25	602696	4.81
Hc6xHe34	400440	3.88	He12xHe34	401169	0.81	He40xHe17	500182	0.16	He42xHe17	401048	5.96	He17xHe25	400440	8.95
Hc6xHe34	700119	1.11	He12xHe34	702357	3.96	He40xHe17	501383	2.60	He42xHe17	400131	9.66	He17xHe25	700119	15.22*
Hc6xHe17	500182	6.31	He12xHe34	602696	0.91	He40xHe17	602696	4.11	He42xHe25	606001	3.29			
Hc6xHe17	400440	0.45	He12xHe33	501785	2.67	He40xHe17	700119	2.37	He42xHe25	401048	6.49			
Hc6xHe17	500182	1.21	He12xHe17	401048	1.41	He40xHe25	400131	1.94	He42xHe25	700119	7.47			
Hc6xHe17	400131	9.48	He12xHe17	401169	0.51	He40xHe25	400440	10.64	He42xHe25	400131	9.68			
Hc6xHe17	700119	3.76	He12xHe17	501785	0.52	He40xHe25	401048	0.40						
Hc6xHe17	702357	3.06	He12xHe17	602696	2.61	He40xHe25	501383	1.73						
Hc6xHe25	400131	10.64	He12xHe17	700119	3.32	He40xHe25	600959	1.30						
Hc6xHe25	700119	15.26*	He12xHe17	702357	3.33	He40xHe25	602696	5.06						
			He12xHe25	401048	12.89*	He40xHe25	700119	4.72						
			He12xHe25	502348	0.67									
			He12xHe25	602696	5.15									
			He12xHe25	702357	3.49									

*Probabilities of Bonferroni's corrections for $\alpha = 0.05$, 0.00047 ($\chi^2_{table} = 12.11$).

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

Pairs of loci	Juveniles (Dbh <48 cm)	Adults (Dbh ≥48 cm)
Hc6xHc12	0.85417	0.02083
Hc6xHc42	1.00000	0.16389
Hc6xHc14	0.81007	0.54931
Hc6xHc40	0.52049	1.00000
Hc6xHc34	0.06181	0.00104
Hc6xHc33	0.01146	0.26250
Hc6xHc17	0.62396	0.63889
Hc6xHc25	0.26285	0.70938
Hc12xHc42	0.14722	0.81076
Hc12xHc14	0.05313	0.74479
Hc12xHc40	0.89514	0.98090
Hc12xHc34	0.06632	0.05660
Hc12xHc33	0.96285	0.79479
Hc12xHc17	0.51840	0.61840
Hc12xHc25	0.43924	0.99688
Hc42xHc14	0.11354	0.82674
Hc42xHc40	1.00000	0.55278
Hc42xHc34	0.11215	0.12951
Hc42xHc33	0.46111	0.28160
Hc42xHc17	0.34931	0.36493
Hc42xHc25	1.00000	0.85104
Hc14xHc40	0.36076	0.25625
Hc14xHc34	0.14826	0.78125
Hc14xHc33	0.16528	0.60938
Hc14xHc17	0.05451	0.50208
Hc14xHc25	0.91563	0.60069
Hc40xHc34	0.34028	0.89861
Hc40xHc33	0.66042	0.61910
Hc40xHc17	0.76389	0.30104
Hc40xHc25	0.74340	1.00000
Hc34xHc33	0.31632	0.00104
Hc34xHc17	0.06389	0.00139
Hc34xHc25	0.32535	0.30972
Hc33xHc17	0.13472	0.77396
Hc33xHc25	0.75486	0.04896
Hc17xHc25	0.64653	0.45799

The values represent the probability of genotypic linkage after 1000 permutations of alleles among individuals. Probability of Bonferroni's corrections: $P = 0.00069$ ($\alpha = 0.05$). Dbh = diameter at breast height.

CONCLUSIONS

The nine microsatellite loci tested adhered to Mendelian inheritance assumptions and showed no linkage or genotypic disequilibrium, indicating that these loci can precisely estimate important population genetic parameters applied to genetic diversity and structure, mating system, and gene flow studies in *H. courbaril*.

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