Molecular Ecology Resources (2008) **8**, 1074–1077 doi: 10.1111/j.1755-0998.2008.02159.x

PERMANENT GENETIC RESOURCES

Isolation and characterization of microsatellite loci for *Hymenaea courbaril* and transferability to *Hymenaea stigonocarpa*, two tropical timber species

A. Y. CIAMPI,* V. C. R. AZEVEDO,* F. A. GAIOTTO,† A. C. S. RAMOS‡ and M. B. LOVATO‡
Embrapa Recursos Genéticos e Biotecnologia, Parque Estação Biológica (PqEB) Avenida W5 Norte (final), CP 02372, CEP 70770-900 Brasília, DF, Brazil, †Universidade Estadual de Santa Cruz (UESC), CEP 45652-050 Ilhéus, BA, Brazil, ‡Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (UFMG), Caixa Postal 486, CEP 31270-901 Belo Horizonte, MG, Brazil

Abstract

Hymenaea courbaril **is a tropical timber species, intensely exploited and found in the Amazon, Atlantic Forest and Brazilian Cerrado biome. Nine highly polymorphic microsatellite loci were developed from a genomic library enriched for AG/TC repeats. In a total of 41 individuals, from two natural populations, seven to 13 alleles per locus were detected and expected heterozygosity ranged from 0.75 to 0.90. Seven loci were effectively transferred to** ***Hymenaea stigonocarpa*****. High levels of polymorphism make the present primers useful for population genetic studies and are a powerful tool to investigate mating system, gene flow and spatial genetic structure.**

Keywords: conservation, genetic diversity, *Hymenaea*, microsatellite, transferability

Received 4 November 2007; revision accepted 22 January 2008

Hymenaea courbaril (Leguminosae, Caesalpinoideae) commonly known as jatobá is a timber and medicinal species that can be found in the Amazon, Atlantic Forest and Brazilian Cerrado biome. This species is listed in the official list of Brazilian endangered medicinal species (IBAMA, Instituto Brasileiro do Meio Ambiente e dos Recursos Renováveis 1992) due to intense anthropogenic activities. *Hymenaea stignocarpa*, an important congeneric vicariant species, occurs in the Cerrado biome. The conservation of genetic resources in tropical biomes is of great importance, since they have been degraded on a large scale in recent decades. Microsatellite markers are important tools for generating detailed pictures of genetic diversity, population genetic structure and to address biogeographical questions. These data, in turn, are useful for the development of strategies for sustainable forest conservation and management practices. We report the development and transferability of highly polymorphic microsatellite loci in the genus *Hymenaea*.

A microsatellite-enriched library was constructed as described by Rafalski *et al*. (1996) adapted by Buso *et al*. (2003). Total genomic DNA was extracted from expanded leaves of a single individual of *H. courbaril* using a cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle & Doyle 1987). DNA was digested with *Sau*3AI and fragments were separated on a 2% agarose gel. DNA between 200 bp and 800 bp was recovered using the QIAquick Gel Extraction kit of QIAGEN, linked to adaptors, amplified and used to construct the enriched genomic library. The fragments were ligated into the pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* XL1-Blue. Positive clones were selected by hybridization with a poly AG/TC probe and sequenced on an ABI PRISM 377 Applied Biosystems (PerkinElmer) automatic fragment analyzer. Primers to the flanking regions were designed using PRIMER3 output software (Rozen & Skaletsky 2000).

Microsatellite loci were amplified by polymerase chain reaction (PCR) in 13 μL containing: DNA (3 ng), 1× PCR reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), forward primer (0.3 μM), and reverse primer (0.3 μM), $MgCl_2$ (1.5 mM), BSA (0.25 mg/mL), dNTP (0.25 mM), *Taq* polymerase (1 U) and ultrapure water. PCR conditions were: denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at T_a (Table 1) for 1 min, and

Correspondence: Dra. Ana Y. Ciampi, Fax: 55 61 33403624; E-mail: aciampi@cenargen.embrapa.br

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Table 1 Nine microsatellite marker loci for *Hymenaea courbaril* (41 individuals) and *Hymenaea stigonocarpa* (40 individuals), across populations

					H. courbaril						*H. stigonocarpa*						
Locus	Repeat array	Primer sequence (5′–3′)	Allele size range	T_a °C	A	H_E	H_O	F_{IS}	$Pr(Ex_1)$	$Pr(Ex_2)$	A	H_E	H_O	F_{IS}	$Pr(Ex_1)$	$Pr(Ex_2)$	Accession no.
Hc06	$(CT)_{28}$	F: AACCGAGTCTCCCTCCATCT R: TGTCACAAGAATAGCAAGGGAG	54–124	60	12	0.85	0.93	–0.095	0.521	0.688	7	0.51	0.26	0.491	0.147	0.317	EU244701
Hc12	$(TC)_{21}$	F: TGTTCCAATTTATGTCCATGGTT R: TGGATGGTTGTGAAGAAAAGG	146–214	60	10	0.83	0.67	0.203	0.487	0.659	7	0.69	0.48	0.319	0.264	0.427	EU244702
Hc14	$(TC)_{17}$	F: CATTCTGCCATCGGTAGGTT R: TCACCCAAACAGGAGTGAA	121–153	58	8	0.83	0.73	0.121	0.474	0.647	5	0.16	0.15	0.105	0.014	0.088	EU244703
Hc17	$(TC)_{13}$	F: TGATTTCATTCCCCTCTTGC R: GGTCAAAGAAAATGCTGGCT	108–130	58	7	0.78	0.48	0.398	0.374	0.608	—	—	—	—	—	—	EU244704
Hc25	$(TC)_{26}$	F: TGCAATTCGACTTCTTGGTT R: AAACACCGATTGACATTGTTTT	110–192	58	13	0.90	0.54	0.410	0.229	0.374	—	—	—	—	—	—	EU244705
Hc33	$(AG)_{16}$	F: GAACAAATCAACTTTCTTTGAAGC R: TTGACGCTTATTTTGCACCA	108–160	58	8	0.75	0.70	0.055	0.368	0.556	7	0.73	0.50	0.319	0.312	0.486	EU244706
Hc34	$(TG)_9(AG)_{12}$	F: CCAGCCCATGACGAAGT R: GGTGTCGTGTTGTGTATGGC	186–220	58	13	0.88	0.73	0.170	0.583	0.738	7	0.84	0.42	0.498	0.491	0.663	EU244707
Hc40	$(AG)_{26}$	F: CCTCTCTCCCAAATTCACGA R: TGCAATAGAATTTCCGAGGC	155–209	60	13	0.81	0.68	0.162	0.450	0.626	7	0.80	0.48	0.393	0.425	0.606	EU244708
Hc42	$(CA)_5T(AG)_{19}$	F: TGGCTAAAAGTTGGGAGGGT R: TTCCCCCTTTTCATGTTGTC	115–171	60	13	0.88	0.72	0.183	0.603	0.753	5	0.46	0.42	0.074	0.112	0.270	EU244709
Mean					10.77	0.836	0.687	0.180	0.998	0.99996	6.43	0.601	0.389	0.355	0.889	0.982	

Allele size range (bp), annealing temperature (T_a °C), total number of alleles per locus (A), expected heterozygosity (H_E), observed heterozygosity (H_O), intrapopulation fixation index (F_{IS}), paternity exclusion probabilities [$Pr(Ex_1)$ and $Pr(Ex_2)$].

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extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. Amplifications were performed with a PTC-200 Peltier Thermal Cycler (MJ Research).

For the polymorphism evaluation, reaction products from 12 adult individuals were separated on 4% polyacrylamide gel and visualized by silver staining. The forward primers of the polymorphic loci were fluorescence labelled and used to analyze 41 adult trees from two natural populations, located at Furnas (21 trees), a protected area, and at the Parque Estadual do Rio Doce (20 trees) both in the state of Minas Gerais, Brazil. The number of alleles per locus, mean observed and expected heterozygosities, intrapopulation fixation index and theta-p estimates were calculated using GDA — Genetic Data Analysis version 1.0 (Lewis & Zaykin 2001). Probabilities of paternity exclusion (Slate *et al*. 2000) were estimated using CERVUS version 3.0, (Kalinowski *et al*. 2007) based on the same sampled trees (Table 1). We also checked for the presence of null alleles using the program MICRO-CHECKER (van Oosterhout *et al*. 2004).

For transferability to *H. stigonocarpa*, polymorphic loci were amplified by PCR using the same conditions as described above. Polymorphism was evaluated using a total of 40 individuals from two natural populations: 20 individuals from Parque Nacional da Chapada Diamantina, Bahia, Brazil and 20 from Parque Nacional da Serra do Cipó, Minas Gerais, Brazil.

Fifty-one clones contained both microsatellite and appropriate flanking regions for primer design. Thirty primer pairs were successfully used to amplify simple sequence repeat (SSR) loci. Nine were polymorphic for *H. courbaril* and of those, seven were transferable to *H. stigonocarpa*. For *H. courbaril*, the number of alleles per locus varied from seven to 13 and expected heterozygosity ranged from 0.75 to 0.90 (Table 1). Eight loci, except Hc06, showed departure from Hardy–Weinberg expectations ($P < 0.005$) and no pairwise disequilibrium was detected. At the population level, two loci (Hc25 and Hc34) and four loci (Hc12, Hc14, Hc25 and Hc42) showed departure from Hardy–Weinberg for Parque Estadual do Rio Doce and Furnas populations, respectively. The first estimate of paternity exclusion probability $Pr(Ex_1)$, when the offspring is sampled but the mother is not was 0.998 for the combined loci. The second estimate, $Pr(Ex_2)$, when both the mother and the offspring are sampled was 0.99996 (Table 1).

The fixation index of the *H. courbaril* populations was 0.100 (Furnas) and 0.201 (Parque Estadual do Rio Doce). The departure from Hardy–Weinberg equilibrium detected in both populations is likely to be due to the presence of null alleles in both populations. In fact, the null allele test (MICRO-CHECKER, van Oosterhout *et al*. 2004) detected that three loci showed a significant rate of null alleles, one (Hc 34) for Furnas population and two (Hc25 and Hc42) for Parque Estadual do Rio Doce. This result indicates that the presence of null alleles may not be a characteristic of the loci but the population. The population analysis also detected, for both species, low but significant (C.I. 99%) difference between populations. The theta-p value between *H. coubaril* populations was 0.08 and between *H. stignocarpa* populations was 0.04.

In *H. stigonocarpa*, a total of seven of the obtained loci were amplifiable and showed high levels of polymorphisms. The mean values for observed and expected heterozygosities were 0.389 and 0.601, respectively. The paternity exclusion probability [$P_r(Ex_2)$], for the combined set of seven loci yielded an estimate of 0.982.

This study shows that these SSR loci allow very precise individual discrimination. The nine microsatellite markers developed exhibited a large number of alleles per locus and high heterozygosity. This suggests that these loci are useful for population genetic studies. We are currently using these markers to investigate questions on genetic diversity, spatial genetic structure, mating system and biogeography in natural populations of *H. courbaril* and *H. stigonocarpa* with the aim of applying scientific knowledge to promote conservation and sustainable management.

Acknowledgements

The authors thank José P. Lemos-Filho and Rosângela L. Brandão, Dr Bruno M. T. Walter and Aécio Amaral for collecting samples and GEF Project for their financial support.

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