Molecular Ecology Notes (2003) 3, 435–437 doi: 10.1046/j.1471-8286.2003.00473.x

PRIMER NOTE

Isolation and characterization of nuclear microsatellite loci in the tropical arboreal palm *Oenocarpus bacaba* (Arecaceae)

N. LEPSCH-CUNHA,*‡ C. A. LUND† and M. B. HAMILTON*†
*Biological Dynamics of Forest Fragments Project, National Institute for Research in the Amazon, Manaus, AM 69011–970, Brazil, †Georgetown University, Department of Biology, Reiss Sciences Building 406, 37th and O Streets NW, Washington, DC 20057-1229, USA

Abstract

Studies of mating patterns are needed to determine how habitat heterogeneity created by deforestation influences tropical plant populations. Eight microsatellite loci were isolated with a subtractive hybridization method for *Oenocarpus bacaba* (Arecaceae), a subcanopy palm tree at the Biological Dynamics of Forest Fragments Project, near Manaus, Brazil. Additionally, two heterospecific loci originally developed for the heart palm *Euterpe edulis* were shown to be variable. Loci averaged 9.6 alleles per locus. Five loci did not fit Hardy–Weinberg expectations with significant deficits of heterozygous genotypes consistent with null alleles. Many loci had high average probabilities of paternity exclusion.

Keywords: Arecaceae, microsatellite, *Oenocarpus bacaba*, palm, population structure

Received 3 April 2003, revision accepted 6 May 2003

Fragmented, managed and secondary growth forests surrounded by pastures are increasingly frequent in tropical landscapes (Chazdon & Coe 1999). For plant species in such heterogeneous habitats, estimating reproductive patterns can furnish information necessary to prevent forest impoverishment by identifying the source and fitness of progeny. *Oenocarpus bacaba* Mart. is an arboreal subcanopy palm species distributed throughout the Amazon, including Brazil, Colombia, Venezuela, Guiana, Suriname, French Guiana and Peru (Balick 1986). The species is monoecious with strong dichogamy, and is pollinated by beetles which feed on pollen (Küchmeister *et al.* 1998). It produces more fruits than any other palm in the central Amazon (Scariot 1999), which are consumed by a wide variety of insects, birds and mammals. *Oenocarpus bacaba* produces a nutritious juice and high quality oil similar in taste and chemical composition to olive oil (Balick 1986). Thus, both *O. bacaba*'s broad ecological role in Amazonian forests and its economic value motivate research to improve its conservation, management and possible silviculture.

Correspondence: N. Lepsch-Cunha. ‡Present address: Ministério da Ciência e Tecnologia, Secretaria de Políticas e Programas de Ciência e Tecnologia, Esplanada dos Ministérios, Bloco E, 2° andar, sala 241, 70067-900 Brasília, DF, Brazil. Fax: + 55 (61) 226 0834; E-mail: ncunha@mct.gov.br

The reproductive behaviour of *O. bacaba* varies among continuous forest, disturbed forest and pasture habitats (Cruz 2001; Lepsch-Cunha 2003). Trees in pastures, second growth forest and along forest fragment edges reproduce earlier, more frequently and (except pasture trees) are more fecund than trees in the interior of fragments or in continuous forest (Lepsch-Cunha 2003). However, trees in these disturbed habitats produce more abortive infructescences and inviable seeds at a greater rate than trees in continuous forest. These habitat-specific differences in reproduction could be caused by pollen limitation and higher rates of biparental inbreeding leading to inbreeding depression. These breeding system changes could in turn impact rates of seed production and seedling fitness with consequences for population recruitment and persistence in different types of habitat.

We developed microsatellite loci for *O. bacaba* in order to evaluate overall population genetic structure and mating patterns in a range of habitats. In addition, these genetic markers will permit us to test for effects of maternal genotype and paternity on the fitness of seedlings experimentally transplanted into pasture, forest fragment and continuous forest habitats. Our work is being conducted at the Biological Dynamics of Forest Fragment Project (BDFFP) near Manaus, Brazil (Gascon & Bierregaard 2001).

The loci described here were isolated following the subtractive hybridization method of Hamilton *et al.* (1999;

see detailed protocol at bioserver.georgetown.edu/faculty/hamilton). Genomic DNA was obtained from frozen leaf tissue ground in liquid nitrogen and extracted with DNeasy plant mini kit (QiaGen) following the manufacturer's instructions. Genomic DNA was digested with *Hae*III, *Nhe*I and *Rsa*I (all from New England Biolabs) to obtain a majority of DNA in the 200 to 1200 base pair size range. After treating digests with mung bean nuclease to create blunt ends and ligation of the SNX linker, streptavidin-bead subtractive hybridization used 30-mer biotinylated oligonucleotides with AC, AG, AAG and CAC repeat motifs in independent reactions. Products of the subtractive hybridizations were amplified by polymerase chain reaction (PCR), dot blotted onto a nylon membrane and hybridized to these same biotinylated oligonucleotides. All products showed hybridization signal above background and were ligated into vector and transformed into competent cells. Plates of colonies for each enrichment sequence were screened by colony lift hybridization. Hybridization-positive colonies were picked and the insert PCR amplified with T7 and T3 primers. PCR products were purified with QiaQuick spin columns (QiaGen), sequenced with both T7 and T3 primers in reactions containing 1.5 μL water, 1 μL template, 0.5 μL primer (1 μM) and 2 μL BigDye terminator reaction ready mix (Applied Biosystems) and electrophoresed on a model 3100 sequencer (Applied Biosystems). The resulting sequences were aligned and edited using SEQUENCHER 4.1 (GeneCodes). Potential primers were identified manually and tested with AMPLIFY 1.2 (Engels 1993).

We purchased primers (Operon Technologies) for cloned loci with a regular pattern of at least 10 repeats and also tested six heterospecific loci (EE2, EE3, EE5, EE8, EE15 and EE43) originally developed for the palm *Euterpe edulis* (Gaiotto *et al.* 2001). We experimented with PCR constituents and cycling parameters in an attempt to amplify a single band in the expected size range. Clone sequencing templates diluted 1000 : 1 were used as positive control templates to verify PCR product molecular weight. PCR reactions contained 1–3 μL of DNA template (DNA concentration was not determined), 2 μL of 10× Thermopol buffer (containing 20 mM $MgSO_4$), 0.2 mM each dNTP, 0.2–0.4 μM of each primer, 0.4 μL of 100 mM $MgSO_4$ (final concentration of 4 mM $MgSO_4$), and 0.4 units of Vent exo-polymerase (New England Biolabs) in a total volume of 20 μL. The thermal cycling profile for all loci was 5 min at 96 °C followed by 30 cycles of 96 °C for 45 s, 59 °C annealing temperature for 45 s and 72 °C extension for 30 s.

Loci were initially tested for polymorphism by running PCR products from 10 to 12 individuals on 4% Metaphor gels (BioWhittaker). For those loci that showed multiple alleles, high performance liquid chromatography purified primers sets were ordered with a forward primer label of 6-FAM, HEX (Operon Technologies), NED, PET or VIC (Applied Biosystems). To score genotypes, PCR products

Table 1 Locus name, primer sequences, repeat motif, observed size range, number of individuals genotyped (N), number of alleles (k), observed heterozygosity (H_O), expected heterozygosity (H_E) average probability of paternity exclusion (P_E) and GenBank Accession no.

Locus	Primer sequences (5′–3′)	Repeat	Size range (bp)	N	k	H_O	H_E	P_E	Accession no.
AC5-3#4	F: ACTGTCTGCAGACAATCGAC R: CTTTTGACACCATTAGTCTGC	CT/GT	202–226	31	12	0.839	0.861	0.540	AY262720
AG5-5#12	F: CCTCATGTTACACACAAAAG R: GTCCTATCAACTAGTCTAGCC	AG	94–126	33	13	0.667*	0.875	0.572	AY262721
AG5-5#6	F: CGGACCAGTGTGGGTGTAAG R: TGGCAGAAAGGAATCTATGCAG	CT	139–153	33	7	0.758	0.818	0.443	AY262722
AG5-5#8	F: GAAGATCATATTGTGCACTCG R: AACTTCTCTGCAATCAGATGCG	TC/CT	235–269	33	9	0.303*	0.753	0.369	AY262723
AG5-7#7	F: TCACAAAACGGAAGAGTGTGC R: GCCAGCTCATTAGACCTAGCC	AG	169–193	33	9	0.758	0.723	0.328	AY262724
AG5-7#9	F: ATGGTTAGTAGTTATGAGGC R: TCTTCATGGTAGATGGCTTG	AG	269–297	31	11	0.500*	0.895	0.611	AY262725
CAC50-T2#2	F: TTTCAGCATGTTTCATGAGCG R: TCATATTTGAACTTTTGGTCCC	CTT/GT	304–344	33	13	0.576*	0.852	0.516	AY262726
AG5-5#1	F: TGGTTATGAATCTTAGCCTC R: AAATCCATGCTCCATAGTCC	GA	169–199	29	11	0.806	0.870	0.554	AY262729
EE3†	F: TTCGCGCACACTGAGAG R: GGTAGCGTTGATTGGTCC	AG	198–208	31	6	0.844	0.784	0.382	AY262727
EE15†	F: CCACACAGACACGCAGATAG R: CCTCATGAAGCATCGACCT	AG	155–177	27	5	0.167*	0.645	0.223	AY262728

*Observed and Hardy–Weinberg expected heterozygote frequencies different ($P < 0.05$).
†Primers originally developed for *Euterpe edulis* by Gaiotto *et al.* (2001).

were electrophoresed along with GeneScan ROX 400 or LIZ 500 molecular marker on a 3100 sequencer and sized using GeneScan 3.7 (all from Applied Biosystems). Genotypes for each locus were determined from 33 adult individuals collected from a population located within the 'km 41' reserve of the BDFFP.

CERVUS 2.0 (Marshall *et al.* 1998) was used to summarize the allele and genotype data (Table 1). Loci showed between 5 and 13 alleles with an average of 9.6 alleles per locus. GENEPOP web version 3.1c (http://wbiomed.curtin.edu.au/genepop/; Raymond & Rousset 1995) was used to test individual loci for deviation from Hardy–Weinberg expected heterozygote frequency. The probability test was employed (option 3, equivalent to a two-tailed hypothesis test) using default values for the Markov chain parameters. Five loci did not fit Hardy–Weinberg expectations with significant deficits of heterozygous genotypes consistent with null alleles. Many of the loci also had high average probabilities of paternity exclusion, which should provide powerful markers for paternity exclusion when seed genotype data from several loci are combined. For example, the combined average probability of paternity exclusion for six loci (AG5-5#1, AG5-7#7, AG5-7#9, AG5-5#6, AG5-3#4 and EE3) is 0.982. These loci will be useful to estimate mating system and conduct paternity analyses in the BDFFP populations of *O. bacaba*.

Acknowledgements

This work was supported by Coordenação e Aperfeiçomento de Pessoal de Nível Superior (CAPES), a National Brazilian Institution for Graduate Studies, Georgetown University, a US National Science Foundation award to MBH (DEB9983014), the Biological Dynamics of Forest Fragments Project, Fundação O Boticário de Proteção à Natureza, World Wildlife Foundation of Brazil Training Program (WWF-Brazil) and Fundação Botânica Margaret Mee. We thank R. Adams for laboratory assistance and Léo Marajó dos Reis for field assistance. The ABI 3100 instrument was supported by an award from the US National Science Foundation (DBI-0100061) and Georgetown University.

References

Balick MJ (1986) The palm — Tree of life: biology, utilization and conservation. *Advances in Economic Botany*, **6**, 1–282.

Chazdon RL, Coe FG (1999) Ethnobotany of wood species in second-growth, old growth, and selectively logged forests of northeastern Costa Rica. *Conservation Biology*, **13**, 1312–1322.

Cruz J (2001) Caracterização morfológica, fenológica e produtividade de *Oenocarpus bacaba* Martius (Arecaceae) em floresta de terra firme e pastagens na Amazônia Central. PhD Dissertation. Instituto Nacional de Pesquisas da Amazônia/Universidade Federal da Amazônia.

Engels WR (1993) Contributing software to the internet: the Amplify program. *Trends in Biochemical Sciences*, **18**, 448–450.

Gaiotto FA, Brondani RPV, Grattapaglia D (2001) Microsatellite markers for heart of palm — *Euterpe edulis* and *E. oleracea* Mart. (Arecaceae). *Molecular Ecology Notes*, **1**, 86–88.

Gascon C, Bierregaard RO (2001) The biological dynamics of forest fragments project: the study site, experimental design, and research activity. In: *Lessions from Amazonia: the Ecology and Conservation of a Fragmented Forest* (eds Bierregaard RO, Gascon C, Lovejoy TE, Mesquita R), pp. 31–42. Yale University Press, New Haven.

Hamilton MB, Pincus EL, DiFiore A, Fleischer RC (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *Biotechniques*, **27**, 500–507.

Küchmeister H, Webber AC, Silberbauer-Gottsberger I, Gottsberger G (1998) A polinização e sua relação com a termogênese em espécies de Arecaceae e Annonaceae da Amazônia Central. *Acta Amazonica*, **28**, 217–245.

Lepsch-Cunha N (2003) Efeito da fragmentação do habitat e do desmatamento no sucesso reprodutivo de uma palmeira amazônica: *Oenocarpus bacaba*. PhD Dissertation. Instituto Nacional de Pesquisas da Amazônia/Universidade Federal da Amazônia.

Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, **7**, 639–655.

Raymond M, Rousset F (1995) GENEPOP Version 1.2.: population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Scariot A (1999) Forest fragmentation effects on palm diversity in central Amazonia. *Journal of Ecology*, **87**, 66–76.