supported by a Discovery Project grant to J.P.E. and F.G-G. from the Australian Research Council. Further support was provided by the School of Animal Biology, University of Western Australia.

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doi: 10.1111/j.1755-0998.2009.02659.x

Characterization of 10 microsatellite markers for the understorey Amazonian herb *Heliconia acuminata*

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Abstract

We characterized 10 microsatellite loci for the plant *Heliconia acuminata* from the Biological Dynamics of Forest Fragments Project (Manaus, Brazil). Markers were screened in 61 individuals from one population and were found to be polymorphic with an average of eight alleles per locus. We found moderate to high levels of polymorphic information content, and observed and expected heterozygosities. All 10 markers are suitable for spatial genetic structure and parentage analyses and will be used for understanding *H. acuminata* dynamics across a fragmented landscape.

Keywords: Amazon forest, forest fragmentation, genetic relatedness, *Heliconia acuminata*, microsatellites, parentage analysis

Received 30 December 2008; accepted 9 February 2009

Heliconia acuminata (Heliconiaceae) is a common understorey species of the nonflooded tropical forest of central Amazonia and the Guyanas (Berry & Kress 1991). *Heliconia acuminata* is a perennial, self-incompatible hermaphroditic species with limited vegetative reproduction (E. M. Bruna and W. J. Kress, unpublished data). The flowers are visited by hermit hummingbirds that 'trapline' from one plant to the next (Kress

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1985) and the seeds of *Heliconia* species are exclusively bird dispersed (Berry & Kress 1991).

Heliconia acuminata has been the subject of a long-term investigation at the Biological Dynamics of Forest Fragments Project (BDFFP), located 70 km north of Manaus, Brazil (Bruna 2003). In the early 1980s, fragments of 1-ha, 10-ha and 100-ha were isolated from surrounding forest by cattle pastures to study the effects of forest fragmentation on Amazonian ecosystems (Laurance *et al*. 2002). In 1997, 5000 m^2 permanent plots were established in continuous forests ($n = 6$), 10-ha ($n = 3$) and 1-ha fragments ($n = 4$) and all *H. acuminata* individuals were monitored to investigate how its population dynamics responds to fragmentation (Bruna 2003). As part of an ongoing project, we selected 10 microsatellite loci to evaluate the spatial pattern of fine-scale genetic structure of *H. acuminata* and to disentangle the contribution of pollen and seed dispersal to plant recruitment in both continuous forest and 1-ha fragments.

Microsatellite libraries were enriched by Genetic Identification Services (GIS, www.genetic-id-services.com/) following Jones *et al*. (2002). Genomic DNA was partially restricted with a cocktail of seven blunt-end cutting enzymes and fragments were subjected to magnetic bead capture (CPG, Inc.). In parallel, libraries were prepared using biotin-CA(15), biotin-AAC(12), biotin-AAG(12) and biotin-ATG(12) as capture molecules. Captured molecules were amplified and restricted with *Hin*dIII to remove the adapters. Fragments were ligated into the *Hin*dIII site of pUC19 and recombinant molecules were electroporated into *Escherichia coli* DH5alpha. Clones were randomly selected and sequenced using the ABI PRISM *Taq* dye terminator cycle sequencing methodology. GIS designed primers using DesignerPCR version 1.03 (Research Genetics, Inc.) from 58 microsatellite-containing clones.

Samples of leaf tissue were collected in 1997 and 1998 from 1-ha fragments ($n = 3$ plots) and continuous forest ($n = 1$ plot) at the BDFFP, and frozen samples (–80 °C) were used for DNA extraction using AutoGenprep 965 robot (AutoGen Inc.). Twelve individuals from the continuous forest were selected to test for amplification of 40 primer pairs and, sequentially, to determine optimal annealing temperature for the successful primers. Polymerase chain reactions (PCR) were performed in a 20-μL volume as follows: 10 ng DNA, 2 μL 10× PCR buffer [670 mM Tris-HCI, pH 8.8, 160 mM $(NH_4)_2SO_4$], 0.8 μL 50 mM $MgCl_2$, 1 μL dNTP (0.25 mM), 1 μL 10 μM unlabelled forward and reverse primers, 0.25 5 U/μL *Taq* polymerase (Bioline USA Inc.). Amplifications were performed in a thermal cycler (MJ Research) using the following conditions: initial denaturation at 95 °C for 4 min, 30 cycles of denaturation at 93 °C for 40 s, 54–64 °C annealing temperature for 40 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 4 min. Reaction products were separated on a 1.5% agarose gel staining with ethidium bromide. Polymorphisms were analysed using the Agilent 2100 Bioanalyser (Agilent Technologies) and DNA 500 LabChip kit.

Out of 40 candidate primer pairs, 14 were selected based on successful amplifications and evidence of polymorphism. Forward primers were end labelled at 5′ end with one fluorescent phosphoramidite (6-FAM or HEX). Markers were screened on 61 individuals selected randomly from a single population from the continuous forest. PCR of 10 μL contained 10 ng DNA, 1 μL 10× PCR buffer, 0.3 μL 50 mM $MgCl_2$, 1 μL dNTP (0.25 mM), 0.2 μL10 μM forward primer and 0.3 μL 10 μM unlabelled reverse primer, 0.03 μL 5 U/μL *Taq* polymerase (Bioline USA Inc.). Thermocycler programmes were the same as described above and specific annealing temperatures for each locus are given in Table 1. Fragments were sized on an ABI PRISM 3130xl DNA Analyser (Applied Biosystems) using ROX-labelled size standard prepared as described in DeWoody *et al*. (2004). Fragments were scored using GeneMapper version 4.0 (Applied Biosystems). An average of 12 homozygote individuals per locus were selected for sequencing using BigDye Terminator (Applied Biosystems) to accurately verify the repeat motif. Four markers with low polymorphism level or inconsistent repeat sequences were excluded. Genotyping error rates were determined by re-extracting and re-running 28 samples (46%) for the 10 markers.

The number of alleles per locus ranged from four to 13 with an average of eight. Polymorphic information content, and expected and observed heterozygosities presented moderate to high levels of variation, from 0.3 to 0.8 (Table 1). All loci were in Hardy–Weinberg equilibrium. Null allele frequencies were generally lower than 0.05; however, Hac-B4 displayed a high frequency of 0.2 (Table 1). The combined non-exclusion probability of all 10 loci was low, 0.0303 for the first parent and 0.0018 for the second parent. All these tests were conducted using Cervus version 3.0.3 (Kalinowski *et al*. 2007). Linkage disequilibrium was determined using GenePop version 4.0.7 (Rousset 2007) and loci were not linked after a Bonferroni correction ($P > 0.001$), indicating that markers are independent. Total genotyping error rate was 2.9%.

Based on our results, all markers will be useful in population-level studies including analysis of individual relatedness and parentage analysis. We are currently using these microsatellite loci to evaluate the effects of forest fragmentation on the fine-scale spatial genetic structure of *H. acuminata*. Ecological and demographic information will complement the current genetic investigation. We anticipate that the integration of these data will provide a more comprehensive understanding of the dynamics of this model system in this experimentally fragmented landscape.

Acknowledgements

We thank David Erickson, Jeffrey Hunt and Ida Lopez for technical assistance and helpful suggestions during the project development.

Table 1 Characterization of 10 microsatellite loci for *Heliconia acuminata*

Locus	Primer sequence 5′–3′	Repeat motif	N	T_a (°C)	Size range (bp)	k	PIC	H_O	H_E	Null allele frequency	GenBank Accession no.
Hac-A103	F: ^{FAM}GCATTGGCTTCCTTTCTC R: ACTTGCTTGGTTCCTGTTG	T_9 ... $(CA)_{13}(GA)$	61	60	233–263	13	0.841	0.869	0.863	–0.0075	FJ644651
Hac-A116	F: ^{FAM}GGTTCTGGAGATTGGAAATG R: GTTGGAGGTGAGTTTAGGACTG	$(TC)_{13}(AC)_{10}(GCAC)_2$ $(AT)_2$	61	57	248–278	10	0.632	0.623	0.663	0.034	FJ644652
Hac-A12	F: ^{HEX}CATCGTCTTTGCTGTAATCTTC R: GTCGTAATGCTTCTTGTGATTG	$(CT)_4(GT)_{13}$	61	60	145–201	12	0.84	0.869	0.862	–0.009	FJ644653
Hac-A5	F: ^{FAM}TGGTCAAATCACCTTTTCAAC R: GGACACCCACTCAGTCAAA	$(AT)_6(GT)_{14}$	61	60	161–175	8	0.684	0.754	0.732	–0.019	FJ644654
Hac-B117	F: ^{HEX}TTGCGACAGTTAAAATGAGTG R: ACATACCCACTGCACGAGTAC	$T(TTG)_7$-TGG-$(TTG)_2$	61	54	199–217	6	0.663	0.623	0.711	0.066	FJ644655
Hac-B4	F: ^{HEX}CCTCCCTTTCCTACCAGTT R: GGACAGCGATAACAAGAAGA	$(GCC)_5(TCC)_5$ ... $(TTG)_4$	61	57	211–217	4	0.425	0.311	0.473	0.204	FJ644656
Hac-B6	F: ^{FAM}AACCAAGACCACCTCCACTC R: AGGAACGAACGGCAGATAAG	$(CAA)_7$	61	62	266–275	4	0.464	0.492	0.514	0.01	FJ644657
Hac-C114	F: ^{HEX}ACCTCCAAAAGGAGTAAAGCTA R: AAGGTAAGGGACTGTCCTACAC	$(AGA)_9$	61	58	217–250	7	0.532	0.607	0.58	–0.035	FJ644658
Hac-C7	F: ^{HEX}GAAGCCTCCATCATCTCTTG R: GGCAGAAACTGAGTGGTGA	$(CTT)_7$	61	57	184–205	7	0.62	0.656	0.662	–0.011	FJ644659
Hac-D1	F: ^{HEX}CGCGAAGAAGATGAAGAGC R: CCCGACAGAAGCCCTAATC	$(ATG)_9$	61	62	158–177	7	0.304	0.295	0.328	0.048	FJ644660
Mean						7.8	0.600	0.609	0.638		

Listed are locus name, sequences for forward (F) with respective fluorescent label (FAM or HEX) and reverse (R) primers, repeat motif, number of genotyped individuals (N), annealing temperature (T_a), size range, number of alleles (k), polymorphic information content (PIC), observed (H_O) and expected heterozygosities (H_E), null allele frequency and GenBank Accession no.

We thank the BDFFP for the logistical support, the IBAMA for the permission to transport biological material from Brazil, and Lee Weigt and the Museum Support Center, Smithsonian Institution, for making the molecular work possible.

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doi: 10.1111/j.1755-0998.2009.02627.x

Development and characterization of 30 polymorphic microsatellite markers for the Atlantic surfclam, *Spisula solidissima* (Dillwyn, 1817)

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Abstract

Thirty polymorphic microsatellite markers were developed for the Atlantic surfclam, *Spisula solidissima*, from an enriched library and characterized in 24 clams from a wild population. The number of alleles ranged from 3 to 16 per locus. The expected and observed heterozygosities ranged from 0.1942 to 0.9238 and 0.0833 to 0.875 respectively. Six loci showed significant ($P<0.05$ after Bonferroni correction) deviation from Hardy–Weinberg equilibrium, probably because of the presence of null alleles. Three primer pairs amplified duplicated loci with two involving tandem mini-satellite repeats. Most of the microsatellite markers developed here should be useful for genetic studies in this species.

Keywords: Atlantic surfclam, fishery, microsatellites, *Spisula solidissima*

Received 16 December 2008; revision accepted 9 February 2009

The Atlantic surfclam, *Spisula solidissima* (Dillwyn, 1817), is a marine bivalve widely distributed along the Atlantic coast of North America. Its geographic distribution stretches from the southern Gulf of St. Lawrence to Cape Hatteras, North Carolina. It is mostly found on mud and sandy bottoms from the intertidal zone to 70 m in depth. It occurs at high densities in certain areas and plays an important role in benthic ecology. The Atlantic surfclam is a popular model bivalve used in studies on fertilization and early development. It is also an important fishery resource and supports an annual landing of about 150 000 metric tons. Studies on the population structure of the Atlantic surfclam have been limited partly because of a lack of genetic markers. Here we report the development and characterization of the first set of microsatellite makers for this species.

Microsatellite markers were developed from an enriched genomic library constructed following the method of Tong *et al*. (2006). Genomic DNA was extracted from ethanol-preserved adductor muscles from 12 individuals with the

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