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TECHNICAL NOTE

Isolation of novel microsatellites for the howler monkey bot fly *Cuterebra baeri* (Diptera: Cuterebrinae)

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Abstract The bot fly C*uterebra baeri* is a host-specific parasite of howler monkeys (*Alouatta* spp.). To explore relationships between populations of these taxa on Barro Colorado Island, Panama, we developed 22 microsatellite loci for *C. baeri*. Twelve of these loci were polymorphic; the mean number of alleles per locus was 3.73 ± 0.3 (range = 2–5), with a mean observed heterozygosity of 0.428 ± 0.052 (range = 0.067–0.683). Overall, variability among the 61 larvae sampled was low, perhaps reflecting the isolation of this island population. Analyses of a subset of these individuals revealed that *C. baeri* larvae parasitizing the same howler monkey were more closely related to each other than were larvae from different monkeys. Future studies will use these loci to explore such host-parasite relationships in greater detail.

Keywords *Cuterebra baeri* · Howler monkey bot fly · Microsatellites · Barro Colorado Island

Cuterebrid bot flies (Oestridae: Cuterebrinae) are a specialized lineage of blow-fly-like Diptera endemic to the New World (Catts 1982). The great majority of species in the subfamily Cuterebrinae belong to the genus *Cuterebra. Cuterebra baeri* is the only member of this genus known to use primate hosts, specifically members of the neotropical genus *Alouatta,* commonly known as howler monkeys (Catts 1982; Milton 1996). The population of mantled howler monkeys (*A. palliata*) on Barro Colorado Island (BCI), Panama, has been the subject of numerous long-term ecological and behavioral studies (e.g., Carpenter 1934; Milton 1980). Detailed life-history observations suggest that parasitism by *C. baeri* is an important source of mortality in this primate population (Milton 1996). Comparative analyses of population genetic structure in these species, including patterns of individual relatedness and dispersal, would generate important new insights into this host-parasite interaction. Microsatellite markers have already allowed the characterization of genetic relationships among howler monkeys on BCI (Milton et al. 2009). Here we present a set of novel di-, tri-, and tetranucleotide microsatellite loci for *C. baeri* that can be used to characterize genetic structure within the parasite component of this interspecific interaction.

We extracted genomic DNA from two *C. baeri* larvae using the DNeasy tissue kit (Qiagen, Valencia, CA). We developed a microsatellite library following a modified version of the protocol of Glenn and Schable (2005). Briefly, DNA was digested with the restriction enzyme *Rsa*I (New England Biolabs), ligated to double-stranded linkers [SimpleX-3F 5′-AAAACGTGCTGCGGAACT-3′ and SimpleX-3R 5′- AGTTCCCAGCACG], denatured, hybridized to multiple biotinylated microsatellite oligonucleotides [$(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$, $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(ACTG)_6$, $AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$], and then captured on magnetic streptavidin beads (Dynal

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Table 1 Novel microsatellite loci developed for *Cuterebra baeri*

Locus	Primer sequence (5′–3′)	Genbank Acc. No.	Motif	N	A[1]	Size range	H_O^2	H_E^3	HWE[4]	PI[5]	PI_{sibs}^6
Cuba2	ACATTTCAGCACCCAGGACA	HQ638026	$(AATG)_9$	59	3	205–201	0.356	0.412	***/N.S.	0.41	0.66
	CAGTCGGGCGTCATCAGCATCAC TGTGGCCATGTA[7]										
Cuba7	CAGGATGGATTTGCAGCAGG	HQ638027	$(GAT)_7$	60	3	139–150	0.683	0.495	**/N.S.	0.35	0.59
	CAGTCGGGCGTCATCACCAAACG CCGTAACCTTTG										
Cuba9	CAGTCGGGCGTCATCAACAGTGGC AACAACGGAAG	HQ638028	$(GAT)_6$	60	3	162–183	0.067	0.065	N.S./–	0.87	0.93
	ACAGGGTGGTATTGGTTCCC										
Cuba31	CAGTCGGGCGTCATCATGGGTTC AGAGAGCTGAC	HQ638029	$(ATCT)_{12}$	60	5	121–137	0.417	0.515	***/N.S.	0.33	0.60
	GCCAATGATCTTCAGGAAACAG										
Cuba33	CCCAACGAGGAAGCAACATC	HQ638030	$(GTT)_8$	61	4	204–219	0.574	0.606	N.S./N.S.	0.22	0.50
	CAGTCGGGCGTCATCACCGGACCA GTACTCTGGAAA										
Cuba38	GCACATAGCAGAGATGGAAAGT	HQ638031	$(ATCT)_8 \ldots (GT)_{12}$	60	5	458–274	0.400	0.651	***/N.S.	0.17	0.48
	CAGTCGGGCGTCATCACCATAAGG AAGCTTGGGAA										
Cuba4	ACTATGGAAGTCTGCCCAGC	HQ638032	$(ACAT)_7$	61	3	361–369	0.230	0.205	N.S./N.S.	0.66	0.81
	CAGTCGGGCGTCATCATGCAATGGA CAGTATGTGTGC										
Cuba50	ACTTGTAGGACCAGATTGAATGAC	HQ638033	$(AC)_{11}$	60	2	258–260	0.417	0.499	N.S./*	0.38	0.60
	CAGTCGGGCGTCATCAGGGAACCA ACCTGAGATG										
Cuba58	CAGTCGGGCGTCATCATCGATTCT TATATTGCACCCAACC	HQ638034	$(AC)_{10}$	60	4	380–386	0.450	0.437	N.S./N.S.	0.35	0.61
	AAGCATCCACCGTGCTTTG										
Cuba59	CAGTCGGGCGTCATCATCGGAAAC TAAGGACGACACA	HQ638035	$(AAC)_6$	60	5	267–279	0.533	0.578	N.S./N.S.	0.21	0.50
	GGCTCTTTCAGCTGCTATGAC										
Cuba68	GTCGTCATATTGAGCACTGGAC	HQ638036	$(AC)_{14}$	60	4	332–346	0.583	0.599	N.S./N.S.	0.23	0.51
	CAGTCGGGCGTCATCACGTTGTT CCTATGGCAGCG										
			Total ± SE	60.09 ± 0.16	3.73 ± 0.30		0.428 ± 0.052	0.046 ± 0.054		6.5×10^{-6}	4.0×10^{-3}

Table 1 continued

Locus	Primer sequence (5′–3′)	Genbank Acc. No.	Motif	N	A[1]	Size range	H_O^2	H_E^3	HWE[4]	PI[5]	PI_{sibs}^6
Monomorphic Loci											
Cuba17	CAGTCGGGCGTCATCAGAGTCGA AATGGAACCAGCC	HQ638038	$(ATGT)_6$	61	1	119	0.000	0.000	–	–	–
	GTGCCGCTTTGTCGAGTTAG										
Cuba23	CAGTCGGGCGTCATCAGCTACTCA ATCCATCATGCCC	HQ638039	$(GAT)_7$	61	1	256	0.000	0.000	–	–	–
	GGTGGAATCTTTGTGCAGGC										
Cuba19	CAGTCGGGCGTCATCAGCCGTTCG CGAGATAGAAAC	HQ638040	$(GTT)_8$	61	1	202	0.000	0.000	–	–	–
	CAAACTTCGATCGGCACCTC										
Cuba44	TGAAGCGCTATGCAAGAAGC	HQ638041	$(GTT)_8$	61	1	211	0.000	0.000	–	–	–
	CAGTCGGGCGTCATCACTGCAAG TGCCGTCTGTATT										
Cuba26	CAGTCGGGCGTCATCAGGATCCG CCAACCTACTATT	HQ638042	$(ACT)_9$	61	1	219	0.000	0.000	–	–	–
	GATCGGGTTCTTCCACGGTA										
Cuba28	CAGTCGGGCGTCATCATCCATCC AACCACAGTCAGG	HQ638043	$(GAT)_{21}$	61	1	224	0.000	0.000	–	–	–
	GCCTTGTCACCAACAACTCC										
Cuba22	CAGTCGGGCGTCATCAGCGCTT GGAGATTGTCTG	HQ638044	$(GTT)_{11}$	61	1	256	0.000	0.000	–	–	–
	TGTGGATTGCAACACAGTCG										
Cuba12	CAGTCGGGCGTCATCACCGCTT TGTCGAGTTAGT	HQ638045	$(ACAT)_{10}$	61	1	338	0.000	0.000	–	–	–
	CGAGTCGAAATGGAACCAGC										
Cuba43	TCTAAGCGTCATAGGAAACCC	HQ638046	$(ACAT)_8$	61	1	346	0.000	0.000	–	–	–
	CAGTCGGGCGTCATCAACGTG CTGCGGAACTACAT										
Cuba3	ACCAGAAGCAATTTGGTGG	HQ638047	$(GAT)_{22}$	61	1	454	0.000	0.000	–	–	–
	CAGTCGGGCGTCATCATGCG GAACTGTTTCACCCTA										

[1] Number of alleles [2] observed heterozygosity [3] unbiased gene diversity [4] Significance of deviations from Hardy–Weinberg equilibrium on BCI including/excluding multiple larvae from the same host (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, prior to Bonferroni correction) [5] Probability of identity [6] Probability of identity for sibs [7] One primer per locus was modified with a 5′ CAG tag to enable universal fluorescent labeling

Biotech). Microsatellite-enriched DNA was retrieved from the beads and amplified via PCR primed with SimpleX-3F. Amplicons were sequenced on a 454 system (titanium chemistry) following standard protocols (Roche 454 Life Sciences, Branford CT). Sequences were subjected to a 3′ quality trim in which only 1 of the last 25 bases of the sequence was ambiguous or had a quality score < 20. CAP3 (Huang and Madan 1999) was then used to assemble sequences at 98% sequence identity using a minimal overlap of 75 base pairs. Sequences were searched for the presence of microsatellites using MSATCOMMANDER v0.8.1 (Faircloth 2008), after which primers were designed with Primer3. One primer from each pair was modified on the 5′ end with an engineered sequence (CAG tag 5′-CAGTCGGGCGTCATCA-3′) to enable use of a fluorescently labeled primer (identical to the CAG tag) during genotyping of larvae.

Forty-eight primer pairs were tested using DNA obtained from eight *C. baeri* larvae. PCR amplifications were performed in a 12.5 μL volume (10 mM Tris pH 8.4, 50 mM KCl, 25.0 μg/ml BSA, 0.4 μM unlabeled primer, 0.04 μM tag labeled primer, 0.36 μM universal dye-labeled primer, 3.0 mM $MgCl_2$, 0.8 mM dNTPs, 0.5 units JumpStart Taq DNA Polymerase (Sigma), and ~20 ng DNA template) using an Applied Biosystems GeneAmp 9700. Touchdown thermal cycling programs encompassing a 10 °C span of annealing temperatures (start temperatures 55–58 °C) were used for all loci. Touchdown cycling parameters consisted of 20 cycles of 96 °C for 30 s, annealing (temperature decreased by 0.5 °C/cycle) for 30 s, and 72 °C for 30 s, followed by 20 cycles of 96 °C for 30 s, 48 °C for 30 s, and 72 °C for 30 s. PCR products were run on an ABI-3130xl sequencer using a Naurox size standard prepared as described in DeWoody et al. (2004), except that unlabeled primers began with GTTT. Results were analyzed using GeneMapper v3.7 (Applied Biosystems). Population genetic statistics were calculated with GENEPOP v4.0 (Rousset 2008) and GENALEX v4.6 (Peakall and Smouse 2006).

Twenty-two of the primer pairs tested produced high quality PCR products and were thus used to assess variability in a larger sample of 61 fly larvae collected from 2003–2009 (57 larvae from BCI, 4 from a mainland sample). Of these, 12 loci were polymorphic. The remaining 10 loci were monomorphic across all 61 of the larvae genotyped. Among the polymorphic loci identified, measures of genetic diversity were generally low (e.g., mean observed heterozygosity $= 0.428 \pm 0.052$; Table 1), perhaps reflecting the physical isolation of BCI for the past ~100 years. No significant linkage disequilibrium was detected among loci (all $P > 0.05$). When all BCI larvae were considered (mainland individuals excluded), 4 loci were found to deviate from Hardy–Weinberg expectations

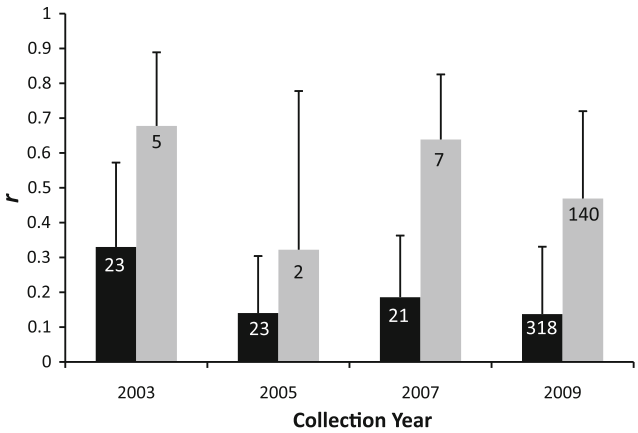

Fig. 1 Mean (±1 SD) pairwise relatedness of bot fly larvae sampled from the same (*gray bars*) versus different (*black bars*) howler monkey hosts. Data from each year sampled were analyzed separately. The number of larvae used in each comparison is shown

(Table 1). Based on visual inspection of data, we suspect that such deviations resulted from our sampling multiple larvae per host (i.e., probable offspring of the same female fly) or from pooling samples over multiple years, rather than from problems with the loci per se (e.g., null alleles). When these analyses were repeated using only 1 larva per host, only 1 locus deviated from HW expectations; after Bonferroni correction of *P* values, no significant deviations from HW expectations were evident (Table 1).

To begin characterizing the genetic structure of *C. baeri* on BCI, we compared coefficients of relatedness (r) among larvae from the same versus different host monkeys; estimates of r were generated using ML-RELATE (Kalinowski et al. 2006), with reference population allele frequencies estimated from the full BCI data set. In all years, r-values for larvae collected from the same host were consistently greater than those for larvae from different hosts (Fig. 1), although at this point statistical analyses would be premature. However, this result is consistent with the hypothesis that multiple larvae on each host often come from eggs laid by the same adult fly, and is a likely explanation for the observed HWE deviations. Together, the markers are capable of distinguishing individuals with high probability (Table 1). Additional sampling of bot flies, including increased sampling of mainland (non-BCI) populations, will provide a more complete picture of genetic variability in this species; the microsatellite loci described here are essential to efforts to understand the genetic dynamics of the relationship between *C. baeri* and its primate hosts.

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