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An Optimized Microsatellite Genotyping Strategy for Assessing Genetic Identity and Kinship in Azara's Owl Monkeys *(Aotus azarai)*

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Abstract
In this study, we characterize a panel of 20 microsatellite markers that reproducibly amplify in Azara's owl monkeys *(Aotus azarai)* for use in genetic profiling analyses. A total of 128 individuals from our study site in Formosa, Argentina, were genotyped for 20 markers, 13 of which were found to be polymorphic. The levels of allelic variation at these loci provided paternity exclusion probabilities of 0.852 when neither parent was known, and 0.981 when one parent was known. In addition, our analysis revealed that, although genotypes can be rapidly scored using fluorescence-based fragment analysis, the presence of complex or multiple short tandem repeat (STR) motifs at a microsatellite locus could generate similar fragment patterns from alleles that have different nucleotide sequences and perhaps different evolutionary origins. Even so, this collection of microsatellite loci is suitable for parentage analyses and will allow us to test various hypotheses about the relationship between social behavior and kinship in wild owl monkey populations. Furthermore, given the limited number of platyrrhine-specific microsatellite loci available in the literature, this STR panel represents a valuable tool for population studies of other cebines and callitrichines.

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Introduction

Azara's owl monkeys *(Aotus azarai)* exhibit a suite of mating and parenting behaviors that is quite rare among primates. They live in small, socially monogamous groups containing a single pair of reproducing adults [Fernandez-Duque, 2011], where the adult male is heavily involved in offspring care, carrying the infant most of the time, and playing, grooming and even sharing food with it [Rotundo et al., 2005; Wolovich et al., 2006, 2008; Fernandez-Duque et al., 2009]. It has long been assumed that *Aotus* males provide care for offspring that they have sired, but this assumption has yet to be formally tested. For this reason, we screened a large number of biparentally inherited autosomal microsatellite markers isolated from a range of primate taxa to identify a set of highly variable loci suitable for delineating genetic identity and assessing parentage in the wild population of *A. azarai* inhabiting our study site in Formosa, Argentina.

Microsatellites, also known as short tandem repeats (STRs), are regions of the genome consisting of sequential repeats (generally 10 or more) of a 2- to 6-base-pair motif that are very likely to differ between any two sampled chromosomes [Pena and Chakraborty, 1994; Morin et al., 1997; Di Fiore, 2003; Kayser et al., 2004]. Although numerous microsatellite loci have been characterized in hominoid and cercopithecoid primates, many of these fail to amplify or display a diminished level of allelic diversity in platyrrhines, which subsequently reduces their efficiency in determining individual identity, parentage, or kinship [Morin et al., 1997, 1998; Witte and Rogers 1999; Lau et al., 2004; Di Fiore and Fleischer, 2004, 2005; Oklander et al., 2007; Raveendran et al., 2008]. In addition, chromosomal fissions, fusions and rearrangements are abundant among platyrrhines, generating variation in their karyotypes even among species of the same genus [Ma, 1981; Ma et al., 1985; Defler and Bueno, 2003, 2007; Stanyon et al., 2003; Katoh et al., 2009]. These facts make it difficult to identify orthologous repetitive regions in disparate *Aotus* taxa and other platyrrhine primates by computational alignment.

As a result, researchers have had to construct platyrrhine species-specific genomic libraries of putative STR-bearing vectors and then sequence and screen these loci in a population sample to identify those sufficiently variable for population genetics studies [Hamilton et al., 1999; Grativol et al., 2001; Böhle and Zischler, 2002; Di Fiore and Fleischer, 2004; Perez-Sweeney et al., 2005; Oklander et al., 2007]. Despite the laboriousness of this approach, the isolation of suitable STR markers for a particular population is still subject to chance, and the genomic positions of markers identified in this manner often remain unknown.

Fortunately, an increasing number of genetic studies involving platyrrhine populations have examined these novel microsatellites across platyrrhine taxa [Ellsworth and Hoelzer, 1998; Nievergelt et al., 1998; Witte and Rogers, 1999; Escobar-Paramo, 2000; Grativol et al., 2001; Böhle and Zischler, 2002; Di Fiore and Fleischer, 2004, 2005; Lau et al., 2004; Amaral et al., 2005; Huck et al., 2005; Perez-Sweeney et al., 2005; Oklander et al., 2007; Muniz and Vigilant, 2008]. Several of these studies include assessments of individuals from the genus *Aotus*. Building on this work, we developed a panel of STR loci that exhibited sufficient allelic variation to enable us to make statistical estimates of kinship between any two individuals in our study population of owl monkeys *(A. azarai)*.

Methods

Samples

The 128 owl monkey samples used in this study were collected from 124 wild individuals in >25 social groups from a wild population of Azara's owl monkeys that inhabits the gallery forests along the Pilagá and Guaycolec Rivers in the province of Formosa, Argentina [Fernandez-Duque, 2009]. Upon capture, each animal was given a physical examination during which hair, blood or tissue samples (ear punches, skin biopsies) were collected for use in genetic analyses [Fernandez-Duque and Rotundo, 2003]. For 1 individual, the source of DNA was the remains of a placenta and fetus found on the ground in the savannah.

In addition, we collected 4 samples from captive individuals of unknown geographic origin at the Saenz-Peña Municipal Zoo, located 250 km away from the study area in the city of Saenz-Peña, Chaco Province, Argentina. The collection of owl monkey samples and related research protocols were approved by the IACUC Committees at the Zoological Society of San Diego and the University of Pennsylvania, and strictly adhere to all legal requirements of both Argentina and the USA. Direct animal contact was crucial for the correct identification of individual owl monkeys, and essential for the precise collection of biological samples for autosomal DNA genotyping.

High-quality DNA was extracted from either tissue or blood using QIAamp DNA Mini and DNA Blood Mini purification kits (Qiagen) according to the manufacturer's protocols. All stock DNA samples were subsequently assayed on a Nanodrop ND-1000 spectrophotometer (Thermo Scientific) to measure DNA quantity (absorbance A at 260 nm) and quality (A_{260}/A_{280} and A_{260}/A_{230} ratios).

PCR Conditions and Microsatellite Genotyping

Following an extensive literature search, we identified a set of microsatellite loci that were isolated from either platyrrhine primates or humans and were known to be variable in at least 1 New World primate species. From this set, we then selected 21 for testing in our study population, all of which had been noted in previous reports as being polymorphic within the set of *Aotus* individuals. All loci were optimized for PCR amplification through the modulation of annealing temperatures and $MgCl_2$ concentrations on a Touchgene gradient thermocycler (Techne) using 'touch-down' PCR cycling parameters [Don et al., 1991; Korbie and Mattick, 2008].

Upon optimization, all samples were genotyped for the STR loci using Geneamp 9700 thermocyclers (ABI). All reactions used Amplitaq Gold polymerase (ABI) with fluorescent forward primers (5′ labeled with 6-FAM dye) and nonfluorescent reverse primers (Sigma Genosys). DNA samples were diluted to 5 ng/μl working concentrations, from which 4 μl (20 ng) of DNA was used per PCR reaction (1 individual at 1 microsatellite locus). Although high-quality DNA samples were used in every experiment, each genotyping reaction was repeated at least 4 times to confirm scoring homozygotes [Morin et al., 2001; Soulsbury et al., 2009]. The specific PCR reagent volumes and cycling conditions for the different loci are listed in table 1.

PCR amplification success was evaluated via standard gel electrophoresis using high-resolution 3% NuSieve agarose (Lonza) and digitally recorded using the Molecular Imaging v4.5.1 software on a Kodak GL200 Imaging Station (Carestream Health). Aliquots of successful PCR amplifications were subsequently combined with 0.3 μl LIZ500 allelic marker standard (ABI) and 8.0 μl Hi-Di formamide (ABI) to make 10 μl final reaction volumes for capillary-based fluorescent fragment analysis. Reactions were denatured at 95°C for 3 min and then immediately placed on ice for 3 min. All reactions were run on a 3130xl Gene Analyzer (ABI) for fragment analysis, and alleles were read and scored using Genemapper ID v3.2 software (ABI).

DNA Sequencing of Microsatellites

Individual alleles of the different STR loci were amplified using unlabeled versions of the same primers from homozygous individuals and then sequenced in order to identify their repeat motif and repeat length in *A. azarai*. Amplicons were first purified by SAP/*Exo*I digestion (New England Biolabs) and cycle-sequenced using Big Dye™ Terminator v3.1 (ABI) sequencing

Table 1. Primers and PCR conditions

Locus	Identified in species	Repeat motif	Primer
1115	*Lagothrix lagotricha*	$(GT)_3(GA)(GT)_5(CT)(GT)_{11}$	1115-F 1115-R
1118	*Lagothrix lagotricha*	$(GA)_2(GT)_{10}$	1118-F 1118-R
113	*Lagothrix lagotricha*	$(GT)_{15}$	113-F 113-R
Ap40	*Alouatta palliata*	$(TG)_4CA(TG)_6$	Ap40-F Ap40-R
Ap68	*Alouatta palliata*	$(TG)_{17}$	Ap68-F Ap68-R
Ap74	*Alouatta palliata*	$(TG)_{19}$	Ap74-F Ap74-R
CJ13	*Callithrix jacchus*	$(CA)_{13}(TC)_{14}$	CJ13-F CJ13-R
CJ14	*Callithrix jacchus*	$(TC)_{21}(CA)_5$	CJ14-F CJ14-R
D13S160	Isolated from *Homo sapiens* and screened in *Aotus* spp.	$(AT)_{10}$	D13S160-F D13S160-R
D15S108	Isolated from *Homo sapiens* and screened in *Aotus* spp.	$(CT)_5(AT)_5$	D15S108-F D15S108-R
D4S411	Isolated from *Homo sapiens* and screened in *Aotus* spp.	$(TA)_{20}$	D4S411-F D4S411-R
D8S275	Isolated from *Homo sapiens* and screened in *Aotus* spp.	$(TC)_{20}$	D8S275-F D8S275-R
Leon30c73	*Leontopithecus chrysopygus*	$(TC)_{25}(AA)(TC)(TG)_{16}$	Leon30c73-F Leon30c73-R
PEPC3	*Cebus apella*	$(GT)_{13}$	PEPC3-F PEPC3-R
PEPC40	*Cebus apella*	$(CA)_{18}(CT)_{14}(CA)_9$	PEPC40-F PEPC40-R
PEPC59	*Cebus apella*	$(GT)_{18}$	PEPC59-F PEPC59-R
PEPC8	*Cebus apella*	$(CA)_{16}$	PEPC8-F PEPC8-R
SB24	*Saguinus bicolor*	$(CA)_{23}$	SB24-F SB24-R
SB38	*Saguinus bicolor*	$(CA)_{19}$	SB38-F SB38-R
SW34D	*Saimiri boliviensis*	$(CA)_{14}$	SW34D-F SW34D-R
SW65B	*Saimiri boliviensis*	$(AT)_5GT(AT)_5(AC)_{18}$	SW65B-F SW65B-R

Data sources: for *Lagothrix lagotricha*, Di Fiore and Fleischer [2004]; *Alouatta palliata*, Ellsworth and Hoelzer [1998]; *Callithrix jacchus*, Nievergelt et al. [1998]; *Homo sapiens* and *Aotus* spp., Lau et al. [2004]; *Leontopithecus chrysopygus*, Perez-Sweeney et al. [2005]; *Cebus apella*, Escobar-Paramo [2000]; *Saguinus bicolor*, Böhle and Zischler [2002] and Huck et al. [2005]; *Saimiri boliviensis*, Witte and Rogers [1999]. T_A = Annealing temperature; TD = touch-down; n.a. = not assessed.

Primer sequence (5′ to 3′)	Size range bp	PCR	T_A °C	$MgCl_2$ mM	Variable in *Aotus*
6*FAM-GCTCATATTCATACATCCCTTGG TTTGCTTGCTCATTCATTGC	179–193	45cyc TD (62–57)	59	1.5	yes
6*FAM-TTTCTCCCTCTCAGATTACCAG CCTTGAGGTTTTTGGGTTCC	172	40cyc TD (57–52)	52	0.5	no
6*FAM-GCAAAACTCCCCTGTGACTG CCCACTCTCCTCCACAAAGG	143	40cyc TD (57–52)	52	0.5	no
6*FAM-CCACGGTGGCAGAGGAGATTT AGAGGCACGAAGACAAGGACA	171	40cyc TD (59–54)	54	0.5	no
6*FAM-TGTTGGTATAATCTTTCCTA ACATACACCTTTGAGTTTCT	164	40cyc TD (57–52)	52	0.5	no
6*FAM-TGCACCTCATCTCTTTCTCTG CATCTTTGTTTTCCTCATAGC	146	40cyc TD (57–52)	52	0.5	no
6*FAM-CAAGGAAACATAAGTGTGGCTC CAGACATTTTAGCCCCTTCC	238	40cyc TD (60–50)	50	0.75	no
6*FAM-CTTGTGACAGTGGGGGAGTT CAAGTGTGAACATCCATGCC	124–158	40cyc TD (66–56)	56	0.75	yes
6*FAM-CGGGTGATCTAAGGCTTCTA GGCAGAGATATGAGGCAAAA	220–242	40cyc TD (60–50)	50	1.5	yes
6*FAM-AGGAGAGCTAGAGCTTCTAT GTTTCAACATGAGTTTCAGA	170–186	40cyc TD (56–51)	51	1.5	yes
6*FAM-AGGCTGTCTTGGCAGAAAT GATGTAATCCTGTGCTATGGC	134-168	40cyc TD (60–50)	50	0.75	yes
6*FAM-AAATCGCTAGAAAATGTCCA TCACACCTGGGAATTAGAAG	137–151	40cyc TD (65–60)	60	1.5	yes
6*FAM-GGACCTGATTGAAGCAGTC TTCCCTGAGAATCTAATGGAG	240–270	40cyc TD (60–50)	50	0.75	yes
6*FAM-CATGGACTGCAATTCAAGCC ACTTCCAGCCTCCAAAACTATG	211–289	40cyc TD (63–58)	58	1.5	yes
6*FAM-GACAGAGCAAGACTCCATCTC GATCAGTAAACACATGTGCAT	160–164	40cyc TD (55–50)	50	1.5	yes
6*FAM-CAGTGGCAACTCTGTAAGGA GTGGAGTCAACATGCAGAGG	256	40cyc TD (59–54)	54	1.5	no
6*FAM-TTCAGGATGCATCAAATGATT TAGCAGTCTATTTAGGTGTTAAT	257–259	40cyc TD (63–58)	58	1.5	yes
6*FAM-ATCTGCCTATCACTTCTTTC CATTTGCTCTGCTCATTCA	118–138	40cyc TD (59–54)	54	1.5	yes
6*FAM-GCCTCAATGGGTTTTAACC AGAACGAGTCTGTATCTTGA	89–145	40cyc TD (55–50)	50	1.5	yes
6*FAM-CATCAAAGGATATTATTATC TACATTTCTGGATACTAGGC	117–143	40cyc TD (61–56)	56	1.5	yes
6*FAM-TGAAGTAATAAAATACATAG ACATTAGGGTCGATGAGTCC	n.a.	n.a.	n.a.	n.a.	n.a.

chemistry. Excess dye terminators were removed with the Big Dye X Terminator™ purification kit (ABI), and the cycle-sequencing products were separated via capillary electrophoresis on a 3130xl Gene Analyzer (ABI). Read quality of chromatograms was assessed using Sequencing Analysis v5.4 software (ABI), and bidirectional sequences were aligned and assembled using Sequencher v4.9 (Gene Codes) and Geneious Pro v5.16 [Drummond et al., 2010]. All sequences generated in this study have been uploaded to NCBI's Genbank nucleotide database (accession No. JN609277–JN609289).

Statistical and Genomic Analyses

Molecular summary statistics were computed using the population genetic software package Arlequin v3.11 [Excoffier et al., 2005]. Individual identity analyses, parentage exclusion probabilities and locus-specific heterozygosity indices were calculated using the software Cervus v3.0.3 [Marshall et al., 1998; Slate et al., 2000].

The physical locations of the STR loci were estimated using the sequenced DNA templates generated from our *A. azarai* samples with the BLAT algorithm on the UCSC Genome Browser to interrogate marmoset (calJac3), rhesus macaque (rheMac2) and human (hg18) chromosomes [Kent et al., 2002; Karolchik et al., 2008]. This analysis was undertaken to identify any two loci that might be genetically linked based on their chromosomal proximity to one another. The chromosomal locations were selected based on nucleotide similarity (>95%) of the entire sequenced amplicons to these different primate genomes.

Results

Of the 21 STR loci that we tested, 20 were optimized for PCR amplification and fragment analysis of *A. azarai* samples, resulting in 40 reliable allele calls for each individual. Of these 20 STR loci, 7 were monomorphic and 13 were polymorphic in the 128 individuals that were genotyped. The panel of 13 polymorphic markers averaged 6.31 alleles per locus. The mean percentage of the 128 individuals genotyped per locus was 95.47%. The average heterozygosity for the panel across loci was 0.45, and the panel exhibited a mean polymorphic information content value (PIC) of 0.40. Locus-specific values are listed in table 2.

The observed heterozygosity values for each locus were compared to their respective expected heterozygosity estimates, and the genotype frequencies for seven out of the thirteen loci did not deviate from those expected under Hardy-Weinberg equilibrium. Four loci, D4S411, D13S108, D8S275 and SW34D, had higher heterozygosity values than expected, whereas SB38 and CJ14 exhibited lower heterozygosity values. In addition, by using Cervus to estimate null allele frequencies at each locus, we calculated that the predicted occurrence of null alleles ranged from –0.21 to +0.31 across the different markers.

When combined, the 13 polymorphic loci provided a parentage exclusionary percentage of 85.2% when both parents were unknown, and 98.1% when one parent was unknown. These percentages increased to 99.9% when both parents were known, 100% for the detection of individual identity, and 99.9% for the estimation of sibling identities. However, at the three loci with complex repeat motifs (CJ13, Leon30c73 and PEPC40), we observed that alleles that had been genotyped as being identical (i.e. equal PCR fragment lengths) possessed different DNA sequences, suggesting the possibility of 'size homoplasy' at these markers [Estoup et al., 1995; Viard et al., 1998; Roeder et al., 2009].

Table 2. Allelic variation and heterozygosity indices for 13 loci from the *A. azarai* panel

Locus	Alleles n	Typed n	H_O	H_E	HWE p	PIC	Null allele frequency	Sequence accession No.
1115	3	106	0.255	0.239	n.s.	0.211	–0.0373	JN609277
CJ14	12	128	0.695	0.737	0.0000137	0.705	0.0284	JN609278
D13S160	9	128	0.859	0.576	<0.0000001	0.483	–0.2146	JN609279
D15S108	4	129	0.349	0.361	n.s.	0.326	0.0185	JN609280
D4S411	11	129	0.822	0.737	0.002	0.69	–0.0669	JN609281
D8S275	7	129	0.481	0.445	0.003	0.415	–0.0653	JN609282
Leon30c73	5	124	0.177	0.198	n.s.	0.183	0.0633	JN609283
PEPC3	4	128	0.406	0.345	n.s.	0.295	–0.0705	JN609284
PEPC40	2	129	0.326	0.397	n.s.	0.317	0.0968	JN609285
PEPC8	2	129	0.380	0.463	n.s.	0.355	0.0966	JN609286
SB24	9	123	0.258	0.331	n.s.	0.318	0.0762	JN609287
SB38	9	96	0.344	0.611	<0.0000001	0.584	0.3078	JN609288
SW34D	5	123	0.602	0.439	0.0000613	0.352	–0.1594	JN609289

The STR loci 1118, 113, Ap40, Ap68, Ap74, CJ13 and PEPC59 were monomorphic (1 allele present) in *Aotus azarai*. In addition, we were unable to reproduce results from PCR and fragment analysis for locus SW65B in *Aotus azarai*. H_O = Observed heterozygosity; H_E = expected heterozygosity; HWE p = significance (p value) in the deviation from Hardy-Weinberg equilibrium; PIC = polymorphic information content; n.s. = not significant.

We did not detect any significant linkage disequilibrium among any loci in the panel. To confirm this observation, we mapped the physical location of each locus by comparing its nucleotide sequence to the genomes of other primates. The BLAT results matched the loci in our panel to a minimum of 11 different marmoset chromosomes, 12 different rhesus macaque chromosomes and 12 different human chromosomes (table 3).

Discussion

Our STR optimized panel provides a novel assay for investigating genetic relatedness and kinship in owl monkeys. Our observations from the field have shown that, although male owl monkeys invest significant resources in parenting [Rotundo et al., 2005; Wolovich et al. 2008], it is possible that these resources may be directed at offspring that they did not sire [Fernandez-Duque et al., 2008, 2009]. Such a finding would represent a clear deviation from the predictions of kin selection and parental investment theories [Hamilton, 1964a, b; Trivers, 1972].

However, testing such scenarios requires the identification of the biological relationships between individuals as delineated by their genetic sequences. Until recently, the lack of platyrrhine STR markers made it difficult to assess genetic relatedness in closely related New World primates, where significant homozygosity often impedes the delineation of individual identity [Lau et al., 2004]. We have shown here

Table 3. Chromosomal localization of STR loci comprising the *A. azarai* panel

Locus	Identified in species	Marmoset (calJac3)	Rhesus macaque (rheMac2)	Human (hg18)
1115	*Lagothrix lagotricha*	chromosome 7	chromosome 9	chromosome 10
1118	*Lagothrix lagotricha*	chromosome 12	chromosome 9	chromosome 10
113	*Lagothrix lagotricha*	chromosome 12	chromosome 20	chromosome 16
Ap40	*Alouatta palliata*	chromosome 1	chromosome 17	chromosome 13
Ap68	*Alouatta palliata*	chromosome 12	chromosome 20	chromosome 16
Ap74	*Alouatta palliata*	chromosome 3	chromosome 5	chromosome 4
CJ13	*Callithrix jacchus*	chromosome 11	chromosome 14	chromosome 11
CJ14	*Callithrix jacchus*	–	–	–
D13S160	*Homo sapiens*	chromosome 1	chromosome 17	chromosome 13
D15S108	*Homo sapiens*	chromosome 10	chromosome 7	chromosome 15
D4S411	*Homo sapiens*	chromosome 3	chromosome 5	chromosome 4
D8S275	*Homo sapiens*	chromosome 16	chromosome 8	chromosome 8
Leon30c73	*Leontopithecus chrysopygus*	chromosome 6	chromosome 12	chromosome 2
PEPC3	*Cebus apella*	chromosome 22	chromosome 19	chromosome 19
PEPC40	*Cebus apella*	chromosome 14	chromosome 13	chromosome 2
PEPC59	*Cebus apella*	chromosome 6	chromosome 7	chromosome 15
PEPC8	*Cebus apella*	chromosome 4	chromosome 4	chromosome 6
SB24	*Saguinus bicolor*	chromosome 3	chromosome 5	chromosome 4
SB38	*Saguinus bicolor*	chromosome 12	chromosome 20	chromosome 16
SW34D	*Saimiri boliviensis*	–	–	–

Data sources: BLAT alignment program [Kent et al., 2002] and the UCSC Genome Browser [Karolchik et al., 2008]. The highly repetitive sequences for CJ14 and SW34D failed to align to a single location for any of the genome assemblies examined. Instead, when 95% sequence identity thresholds in BLAT were applied, these two loci mapped to many different genomic locations at low confidence levels.

that this panel of 13 variable STRs can estimate paternity and kinship with a high degree of certainty among owl monkey individuals, thereby permitting further exploration of the social structure of this monogamous taxon.

However, complex STR repeat motifs should be carefully considered in any parentage assessment, as 'size homoplasy' could skew the delineation of parental alleles from the interpretation of fragment size alone. For example, the amplification of a locus that possesses two or more distinct repeat motifs, such as Leon30c73 (CT_{18} + GT_{13}), could potentially generate allelic fragments of the same length in 2 individuals that have different combinations of the 2 repeat motifs. Occurrences of such size homoplasies have been noted in other STR panels that take advantage of cross-specific amplification of microsatellite loci [Roeder et al., 2009; Soulsbury et al., 2009]. For this reason, we recommend the direct sequencing of these regions when they are to be used for pedigree or population studies. Nevertheless, our microsatellite panel possesses sufficient exclusionary power for the discernment of individual identity among *Aotus* individuals. It may further prove to be valuable for population and sociobehavioral studies of other cebine and callitrichine taxa.

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