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BRIEF REPORT

A Suite of Microsatellite Markers Optimized for Amplification of DNA From Addax (*Addax nasomaculatus*) Blood Preserved on FTA Cards

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The addax (*Addax nasomaculatus*) is a critically endangered antelope that is currently maintained in zoos through regional, conservation breeding programs. As for many captive species, incomplete pedigree data currently impedes the ability of addax breeding programs to confidently manage the genetics of captive populations and to select appropriate animals for reintroduction. Molecular markers are often used to improve pedigree resolution, thereby improving the long-term effectiveness of genetic management. When developing a suite of molecular markers, it is important to consider the source of DNA, as the utility of markers may vary across DNA sources. In this study, we optimized a suite of microsatellite markers for use in genotyping captive addax blood samples collected on FTA cards. We amplified 66 microsatellite loci previously described in other Artiodactyls. Sixteen markers amplified a single product in addax, but only 5 of these were found to be polymorphic in a sample of 37 addax sampled from a captive herd at Fossil Rim Wildlife Center in the US. The suite of microsatellite markers developed in this study provides a new tool for the genetic management of captive addax, and demonstrates that FTA cards can be a useful means of sample storage, provided appropriate loci are used in downstream analyses. Zoo Biol 31:98–106, 2012. © 2011 Wiley Periodicals, Inc.

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INTRODUCTION

The addax (*Addax nasomaculatus*) is a critically endangered antelope, currently found in isolated areas of the Sahara desert [Newby and Wacher, 2008]. Due, in part, to the conservation status of the species, a portion of the captive population in North America is managed through a Species Survival Plan® (SSP). The Addax SSP produces regular breeding recommendations for the captive population to ensure that the population remains both demographically and genetically healthy. The Addax SSP also participates in the conservation of wild populations by periodically providing animals for reintroduction, as was most recently done in Tunisia as part of an ongoing national recovery strategy for Tunisian wildlife.

Traditional SSPs use pedigree analyses to develop breeding recommendations designed to retain maximal levels of gene diversity while limiting inbreeding [Ballou and Lacy, 1995; Foose and Ballou, 1988; Hedrick and Miller, 1992; Lacy, 1994]. However, genetic management is hampered if a population's pedigree is only partially known because the relationships among individuals are unclear and genetic analyses are inaccurate [Ballou and Lacy, 1995]. Incomplete and unknown pedigrees currently impede the ability of the Addax SSP to confidently manage the genetics of the captive population and to select appropriate animals for reintroduction.

In the absence of complete pedigrees, molecular markers can be utilized to establish familial relationships among individuals. Microsatellites are ideal markers for revealing genetic relationships among individuals because of their co-dominant inheritance pattern and high level of variability [Goldstein and Schlotterer, 1999; Sunnucks, 2000]. Thus, microsatellite analysis could be a powerful tool to improve the long-term effectiveness of the Addax SSP by providing more accurate estimates of gene diversity that can be used to improve breeding recommendations.

A more general consideration when using molecular data to improve the genetic management of captive populations is the DNA source. Often, blood samples for genetic analysis are collected opportunistically (e.g., during annual physical exams) across a large number institutions. This can result in collection dates that vary by a year or more, during which time samples can become degraded due to temperature variation and spoilage. Extracted DNA or stored tissue may also degrade in the laboratory, resulting in problems for re-analysis of samples. Efforts to reduce this potentially important source of degradation have included storage of biological samples on commercially available storage devices specifically designed to provide long-term storage of DNA at room temperature. For captive populations, long-term storage of DNA at room temperature would be ideal for simplifying sample collection and shipping, as well as mitigating degradation over time and minimizing both laboratory space and frozen storage requirements. One such technology, FTA® Cards (Whatman, Ltd.), consists of filter paper embedded with a proprietary mix of chemicals that serve to lyse cells,

prevent bacterial growth, and protect DNA in the sample. Although these cards have been used in a variety of applications, their utility under field conditions may be reduced compared to conventional storage methods [Borisenko et al., 2008]. Thus, the goal of this study is to develop a suite of microsatellite markers that are ideally suited to genotype captive addax from blood samples stored on FTA cards.

METHODS

Thirty-seven blood samples were collected opportunistically from addax at Fossil Rim Wildlife Center. A total of 250 μL of whole blood (~1/4 cc) was taken from each animal and spotted on FTA cards. The blood was allowed to air-dry and then cards were stored at room temperature for up to 6 months until processing. DNA was prepared for analysis following the manufacturer's recommended protocols. First, a 1.2 mm punch was taken from the dried blood spot on the filter paper. This punch was washed with FTA Purification Reagent, then with TE^{-1} buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). DNA on the washed punch was then ready for PCR.

We tested amplification for 66 microsatellite markers previously described in other ungulate species, including 14 known to be polymorphic in addax [Armstrong et al., 2010]. PCR was performed in 10 μL reactions containing the washed punch, 0.2 mM each dNTP, 5 pmol forward and reverse primers, 1.5 mM $MgCl_2$, and 1 Unit Taq DNA polymerase in 1 × PCR buffer. PCRs were performed using the following thermocycler profile: an initial denaturation step at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, Ta (annealing temperature, Table 1) for 30 s, and 72°C for 30 s, then a final extension at 72°C for 5 min. Reagent concentrations and thermocycler profiles were varied during locus optimization. PCR products were visualized on 1% agarose gels stained with ethidium bromide.

Loci that reliably amplified a single product in the expected size range were further screened for levels of polymorphism on an ABI 3730xl DNA Analyzer at the University of Wisconsin Biotechnology Center. For these PCRs, conditions were identical to those for locus optimization described above, with two exceptions: (1) the final extension step was changed to 60°C for 45 min, as we have found that this dramatically reduces allele "stutter", and (2) 0.5 μL

TABLE 1. Amplification Conditions and Genetic Diversity Estimates Based on 37 Captive Addax

Locus	Ta	Size	♯ alleles	He	Ho	*P* (HWE test)
RT24	58	330–340	3	0.635	0.548	0.4762
BM848	60	225–240	6	0.599	0.656	0.0016
C273	56	190–260	3	0.588	0.000	<0.0001
OarFCB48	54	150–170	4	0.714	0.583	0.0292
ETH225	54	150–160	2	0.131	0.139	1.0000

Annealing temperature (Ta), approximate size of the PCR product (Size), the number of alleles per locus (♯ alleles), expected (He) and observed (Ho) heterozygosity values, and *P*-values for Fisher's exact tests for deviations from Hardy–Weinberg equilibrium are reported.

of fluorescent marker (5′-CAGTCGGGCGTCATCA-3′ labeled with either HEX or 6-FAM; Schuelke, 2000) was added to each PCR to enable visualization on the ABI 3730xl. Alleles were sized using an internal lane size standard in GeneMarker 1.91 (Softgenetics). GDA software [Lewis and Zaykin, 2001] was used to calculate basic genetic diversity statistics, including numbers of alleles per locus, observed (H_o) and expected (H_e) heterozygosity, and Fisher's exact tests for deviations from Hardy–Weinberg expectations (based on 5,000 shufflings). To assess the power of this suite of microsatellite loci, we calculated an unbiased probability of identity (P_{ID}) and combined probability of exclusion (P_E) in CERVUS 3.0 [Marshall et al., 1998; Kalinowski et al., 2007].

RESULTS

Sixteen of the 66 markers (24%) reliably amplified a single product within the expected size range. Only 5 of the 16 markers (31%) were found to be polymorphic in addax from the Fossil Rim Wildlife Center population. The remaining 11 loci amplified a product of the expected size range, suggesting that a repeat-containing microsatellite was indeed amplified; however, these loci were not found to be polymorphic in our sample of captive addax. Based on the five polymorphic loci, levels of polymorphism were low across 37 captive addax (Table 1). Numbers of alleles per locus ranges from 2 to 6, and heterozygosity ranged from 0.131 to 0.714. Observed heterozygosities were less than expected at almost all loci, and deviated from Hardy–Weinberg expectations at three loci (BM848, C273 and OarFCB48; Table 1). This is not entirely surprising, given that our sample included a small number of animals from only one herd. It is possible that locus C273 has null alleles, since we did not observe any heterozygous genotypes. However, it is difficult to unambiguously distinguish null alleles from other demographic processes such as nonrandom mating using allele frequencies alone. Alternatively, it may be that locus C273 is linked to a locus under selection in captive addax; however, this seems unlikely given that locus C273 is not linked to loci under selection in other ungulate species. Regardless of the underlying cause, a lack of heterozygotes at locus C273 warrants caution before employing this marker in a larger study of addax. This suite of microsatellite loci offers a probability of identity (P_{ID}) of 0.000979, meaning that fewer than 1 in 1,000 unrelated animals will share the same multilocus genotype. For paternity testing, this suite of microsatellites offers a probability of exclusion (P_E) of 0.8365, suggesting that fewer than one in six males will falsely appear to be a father of an offspring with unknown paternity.

DISCUSSION

In a recent study of cross-species microsatellite amplification in addax, 75% of ovine, bovine, and caprine microsatellite loci successfully amplified in captive addax [Armstrong et al., 2010]. This is exciting for the genetic management of addax, and suggests that powerful genetic analyses of addax are possible. In our study, we focused on microsatellite loci that could be reliably amplified from blood stored on FTA cards. Although there are a number of potential

benefits associated with the use of FTA cards for sample storage, it is important to select molecular markers for downstream analysis that are well-suited to this medium. We identified five polymorphic microsatellite markers that are ideally suited to amplify DNA from captive addax samples preserved on FTA cards.

Our cross-species amplification success rate (24%) for DNA extracted from FTA cards was far lower than that reported for DNA extracted from hair follicles [75%; Armstrong et al., 2010], and loci that successfully amplified DNA from hair follicles [Armstrong et al., 2010] were not successful in amplifying DNA from FTA cards. These results suggest that microsatellite loci may not be equally efficient at amplifying DNA from different sources, and similarly that the optimal suite of microsatellite loci for a given study may depend on the method of sample collection [Taberlet et al., 1999; Beja-Pereira et al., 2009]. Our finding of 31% polymorphic loci is similar to the percentage of polymorphic loci identified by Armstrong et al. [2010]. It is possible that the small number of polymorphic loci identified in both studies is due to cross-species amplification itself, whereby loci from distantly related taxa are less likely to amplify product than loci from closely related taxa [e.g., Primmer et al., 1996; Luikart et al., 1999; Garner et al., 2005]. However, the polymorphic loci we identified were not only those originally characterized in Cervidae, but also included loci originally characterized in Bovidae (cattle) and Ovidae (sheep). Polymorphic loci identified in Armstrong et al. [2010] were originally characterized in Bovidae and Ovidae (none from Cervidae). Thus, it seems more likely that the small number of polymorphic loci identified in addax is a direct result of population history. A severe population bottleneck associated with the founding of the sampled captive population, followed by generations of captive breeding, has likely resulted in a low level of polymorphism in the species.

In our sample of 37 captive addax, we observed relatively low levels of polymorphism. Despite the overall low level of polymorphism, we obtained a high level of resolution with relatively few markers. These results suggest that this suite of loci can be used for individual identification or paternity analysis for relatively little cost. Thus, this suite of microsatellite loci that can reliably amplify DNA preserved on FTA cards provides a new and important tool for future genetic management of captive populations of addax.

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APPENDIX A

Information for all loci attempted in this study. The locus name, Gen Bank accession number, primer sequences used, and original references are provided in Table A1.

TABLE A1.

Locus	GenBank	Primer sequence	Reference
P	AF102240	F: TTTCACTGTTTTCTCCTTCAGA R: TGCCCAATCAGATGTTGTAG	Jones et al. [2000]
Q	AF102241	F: AATGTGTCAGTGAAGGTCTTC R: ATCCAGGCAACCATCTAG	Jones et al. [2000]
K	AF102242	F: GCAGGAAGGAGGAGACAGTA R: GCTGGTTCGTTATCATTTAGC	Jones et al. [2000]
R	AF102243	F: GGGGTCTTCTCAATCCA R: TCAGTTTCTGGAACTCTAAAGT	Jones et al. [2000]
O	AF102245	F: ACGAGGTTCAGTGGTTCC R: CAGGGCATAGTTTCCAAA	Jones et al. [2000]
D	AF102247	F: AGAGCCTCGTCTTTTCATTC R: TTGCTGCTTGCTTGTCTAAT	Jones et al. [2000]
A	AF102248	F: GCACATGCTGCAGATTCATT R: GGAGAGAGACCTTGGGAGAAA	Jones et al. [2000][a]
B	AF102249	F: GGAGAGAGCAGGGCATGTTA R: CCCCAAAAGGTCACAAGAAA	Jones et al. [2000][a]
C	AF102250	F: TTCCATCCATCTTGCCATTCA R: TTGGAGGCATGTAGGAAAGG	Jones et al. [2000][a]
E	AF102251	F: CAGGGCAGTGTATCAGTGAGG R: CCAGCCTTCCTGGACTAGAG	Jones et al. [2000][a]
F	AF102252	F: TCACAGGCGATCAAGAAATG R: GGCTTCTGGATGGAGAACCTA	Jones et al. [2000][a]
G	AF102253	F: ACCCTATGGTCACAGCAACA R: CTCTGGGCATCCATGAAGTAG	Jones et al. [2000][a]
H	AF102254	F: TGCTGCCATTGCCAGATAAT R: TCCCAGAGGGCAGATGTCTA	Jones et al. [2000][a]
I	AF102255	F: CATGGTGGGCATTCAGTAGA R: CATAAGGCAGAGAAGGCATTG	Jones et al. [2000][a]
J	AF102256	F: ACGCAACCACTCATTTACCC R: TATGTGCAGAGATGGGTGGA	Jones et al. [2000][a]
L	AF102257	F: CCCTGTGGTCTAGCAAATTCA R: CATGCTCATAAGTGAAGGGATG	Jones et al. [2000][a]
RT1	AY514441	F: TGCCTTCTTTCATCCAACAA R: CATCTTCCCATCCTCTTTAC	Wilson et al. [1997]
RT5	AY514440	F: CAGCATAATTCTGACAAGTG R: AATTCCATGAACAGAGGAG	Wilson et al. [1997]
RT7	U90737	F: CCTGTTCTACTCTTCTTCTC R: ACTTTTCACGGGCACTGGTT	Wilson et al. [1997]
RT9	U90738	F: TGAAGTTTAATTTCCACTCT R: CAGTCACTTTCATCCCACAT	Wilson et al. [1997]
RT10	U90740	F: GAAAAGCAAAGACCCAACAG R: TAACACAGCAAAAGCTTATA	Wilson et al. [1997]
RT13	U90741	F: GCCCAGTGTTTAGGAAAGAAG R: CATCCCAGAACAGGAGTGAG	Wilson et al. [1997]
RT20	U90742	F: GCAGAAGAGTGAGTGTGAGT R: CTGTTGTATTTTGGACCTTT	Wilson et al. [1997]
RT24	U90743	F: TGTATCCATCTGGAAGATTTCAG R: CAGTTTAACCAGTCCTCTGTG	Wilson et al. [1997]
RT25	U90744	F: TGCCAAGGAACCAAGATGTC R: CATTCCAGTATTATTGCCTGA	Wilson et al. [1997]
RT27	U90746	F: CCAAAGACCCAACAGATG R: TTGTAACACAGCAAAAGCATT	Wilson et al. [1997]
RT30	U90747	F: CACTTGGCTTTTGGACTTA	Wilson et al. [1997]

TABLE A1. Continued

Locus	GenBank	Primer sequence	Reference
		R: CTGGTGTATGTATGCACACT	
BL25	U90748	F: AACAGTGGCAATGGAAGTGG R: AGTCAGGATCTAGTGGGTGAGTG	Bishop et al. [1994][b]
BM203	U90749	F: GGGTGTGACATTTTGTTCCC R: CTGCTCGCCACTAGTCCTTC	Bishop et al. [1994][b]
BM415	G18413	F: GCTACAGCCCTTCTGGTTTG R: GAGCTAATCACCAACAGCAAG	Bishop et al. [1994][b]
BM4208	G18500	F: TCAGTACACTGGCCACCATG R: CACTGCATGCTTTTCCAAAC	Bishop et al. [1994][b]
BM6438	G18413	F: TTGAGCACAGACACAGACTGG R: ACTGAATGCCTCCTTTGTGC	Bishop et al. [1994][b]
BM6506	G18509	F: GCACGTGGTAAAGAGATGGC R: AGCAACTTGAGCATGGCAC	Bishop et al. [1994][b]
BM848	G18435	F: TGGTTGGAAGGAAAACTTGG R: CCTCTGCTCCTCAAGACAC	Bishop et al. [1994][b]
BovPRL	G18455	F: GGAAAGTGAACATGACTGTCTAG R: GCCCTCTCTTCTACAATGAACAC	Moore et al. [1994][b]
ETH152	G18477	F: AGGGAGGGTCACCTCTGC R: CTTGTACTCGTAGGGCAGGC	Steffen et al. [1993][b]
ILSTS011	L23485	F: GCTTGCTACATGGAAAGTGC R: CTAAAATGCAGAGCCCTACC	Brezinsky et al. [1993][b]
INRA011	Z14040	F: CGAGTTTCTTTCCTCGTGGTAGGC R: GCTCGGCACATCTTCCTTAGCAAC	Vaiman et al. [1994][b]
OarFCB193	L23485	F: TTCATCTCAGACTGGGATTCAG-AAAGGC R: GCTTGGAAATAACCCTCCTGC-ATCCC	Buchanan and Crawford [1993][b]
OCAM	X12672	F: CCTGACTATAATGTACAGATCCCTC R: GCAGAATGACTAGGAAGGATGGCA	Moore et al. [1992][b]
OvirA	L01533	F: CACAAAGAATCAGACGTGGT R: GTGCATCTCAACATGAGTTAGG	DeWoody et al. [1995]
OvirH	–	F: AAGTCTACAATCCATGGGCTTGC R: GTTCTTTACCACCTGCACCA	DeWoody et al. [1995]
C02	L35576	F: CCTACATCTTCCACCAGACT R: ACAGCAGCCTCCAAGTG	Meredith et al. [2005]
C32	L35583	F: ACAACTGTGTGAGCCAATAC R: AGCAAGTGAAGAAGAATGTTC	Meredith et al. [2005]
C36	AF442813	F: GCTTGAGTGGTGGACTT R: CTCCTGGCTGATAGTAAGAT	Meredith et al. [2005]
T40	AY514436	F: GTTGGGCTTATGAATCAGG R: GCACATCTTTGGACCTCTC	Meredith et al. [2005]
C105	AY514437	F: GGCAGTTCTTGAGGCAAGTC R: AGGTCCTCTAAGGGCAGGAA	Meredith et al. [2005]
T107	AF102244	F: ACATCCGTTCAGGTGTGA R: CCAGAGGTAAGATAAATGGTGA	Meredith et al. [2005]
T115	AF193019	F: AATGTCTGACTCTAGGTGAGTG R: TTTGCTATCTGAGCCACTAG	Meredith et al. [2005]
T267	AF193021	F: ATTCGTGATTGTCTTGTGTC R: TGGACCTCTCAGTAGTTATGAT	Meredith et al. [2005]
C273	AY514439	F: AGGGAAACCTCTGTTCAGGA R: ACCAAGCAAAATGCCTTACA	Meredith et al. [2005]
C276	AF102246	F: AACTGAGAAGCTGCCTGCAT R: ATGGCAAGGGCTCAGAGAT	Meredith et al. [2005]

TABLE A1. Continued

Locus	GenBank	Primer sequence	Reference
BM1818	G18391	F: AGCTGGGAATATAACCAAAGG R: AGTGCTTTCAAGGTCCATGC	Bishop et al. [1994]
HEL13	X65207	F: TAAGGACTTGAGATAAGGAG R: CCATCTACCTCCATCTTAAC	Bishop et al. [1994]
ETH225	Z14043	F: GATCACCTTGCCACTATTTCCT R: ACATGACAGCCAGCTGCTACT	Steffen et al. [1993]
INRA5	–	F: CAATCTGCATGAAGTATAAATAT R: CTTCAGGCATACCCTACACC	Vaiman et al. [1994]
BM6526	G18454	F: CATGCCAAACAATATCCAGC R: TGAAGGTAGAGAGCAAGCAGC	Bishop et al. [1994]
HSC	–	F: CTGCCAATGCAGAGACACAAGA R: GTCTGTCTCCTGTCTTGTCAT	Baumung et al. [2006]
BM1329	G18422	F: TTGTTTAGGCAAGTCCAAAGTC R: AACACCGCAGCTTCATCC	Bishop et al. [1994]
OarFCB48	L20006	F: GAGTTAGTACAAGGATGAC-AAGAGGCAC R: GACTCTAGAGGATCGCAAAGA	Buchanan and Crawford [1993]
OarCP49	OAU15702	F: CAGACACGGCTTAGCAACTAAA CGC R: GTGGGGATGAATATTCCTTCA	Ede et al. [1995]
D5S2 (ETH2)	Z22743	F: TACTCGTAGGGCAGGCTGCCTG R: ACATGACAGCCAGCTGCTACT	Solinas Toldo et al. [1993]
MAF209	M80358	F: GATCACAAAAAGTTGGAT ACAACCGTGT R: TCATGCACTTAAGTATGTAGG	Buchanan and Crawford [1993]
OarFCB11	L01531	F: GGCCTGAACTCACAAGTTGATAT ATCTATCAC R: GCAAGCAGGTTCTTTACCACT	Buchanan and Crawford [1993]
OarFCB20	L20004	F: AAATGTGTTTAAGATTCCA TACATGTG R: GGAAAACCCCCATATATACCT	Buchanan and Crawford [1993]

[a]Loci originally described in Jones et al. [2000] but primers redesigned for this study.

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