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RESEARCH ARTICLES

Cross-Species Amplification, Non-Invasive Genotyping, and Non-Mendelian Inheritance of Human STRPs in Savannah Baboons

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Twenty-nine human microsatellite primer pairs were screened for their utility in the cross-species amplification of baboon DNA derived from both blood and feces as part of a larger study to identify paternal half sisters in a population of wild baboons (*Papio cynocephalus*). Forty-one percent (12/29) of the human primers successfully amplified baboon DNA. Of these 12 primers, six amplified fragments that were both polymorphic and heterozygous (mean number of alleles = 6, mean heterozygosity = 87%) and yielded repeatable results. However, only five of these six simple tandem repeat polymorphisms (STRPs) showed patterns of Mendelian inheritance (i.e., mothers and offspring shared at least one allele at each locus), and were therefore useful for determining relatedness between individuals. Analysis of the sixth primer revealed non-Mendelian inheritance, i.e., three of the six known mother-daughter pairs had no shared alleles. This failure was probably due to non-specific fragment amplification, and may have resulted from a different STRP locus being amplified in mother and daughter. This finding highlights the importance of sampling DNA from known parent-offspring pairs when screening microsatellite primers for genetic studies. Multiple, independent replications of genotypes and Mendelian checks are both particularly important when using cross-species amplification or when using a low-quality source of DNA. Am. J. Primatol. 51:219–227, 2000.

Key words: cross-species amplifications; STRP; Mendelian checks; feces-derived DNA; parent-offspring validation; micro-satellite DNA

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INTRODUCTION

Confirming the accuracy of microsatellite analysis becomes more critical as increasingly exacting information is demanded of DNA from lower-quality sources such as feces. Furthermore, because of the costs associated with cloning species-specific primers, primers developed in humans are increasingly being used to amplify microsatellites, or simple tandem repeat polymorphisms (STRPs) in non-human primates. Some STRP priming sites are highly conserved between humans and many non-human primate species [for a review, see Coote & Bruford, 1996; also see, for apes: Washio, 1992; Gerloff et al., 1995; for Old World monkeys: Morin & Woodruff, 1992; Rogers, 1992; Constable et al., 1995; Altmann et al., 1996; Kayser et al., 1996; Launhardt et al., 1998; von Segesser et al., 1999; for New World monkeys: Witte & Rogers, 1999].

Although cross-species amplification is convenient, it may lead to low or incomplete amplification due to as little as a single dinucleotide mismatch between the primer and the target DNA sequences. This problem can often be resolved by employing less stringent polymerase chain reaction (PCR) conditions (e.g., lowering the annealing temperature or increasing the magnesium concentration in the reaction), but the consequence may be non-specific amplification of a second locus. If this occurs, individuals may exhibit three or more "alleles" with a single primer set because more than one locus is inadvertently being amplified.

This difficulty is compounded when the DNA source is a low-quality one such as feces, an increasingly common situation in studies of wild populations. Allelic dropout, the preferential amplification of only one of two alleles, is the most common and troublesome error associated with poor quality DNA, such as that derived from feces [Gerloff et al., 1995; Kohn et al., 1995; Taberlet et al., 1997; Launhardt et al., 1998]. When allelic dropout occurs, a true heterozygote appears to be a homozygote, resulting in mistyping at the individual level, and an excess of homozygous genotypes at the population level.

When researchers employ cross-species primers to amplify low-quality DNA, they run the risk of encountering the combined effects of non-specific amplification and allelic dropout. These combined effects may actually counter each other, making it very difficult to correctly assign genotypes.

Because of these problems, internal validation checks are especially important when amplifying low-quality DNA with cross-species STRP primers. Genotyping results can be verified by: 1) using the DNA of known individuals [Gerloff et al., 1995; Launhardt et al., 1998], and 2) using a high-quality source of DNA as a species control [Kohn et al., 1995; Paxinos et al., 1997; Wasser et al., 1997]. Using DNA from known individuals controls for intra-subject inconsistencies, ensuring that genotypes amplified from the same genomic DNA sample yield consistent, reliable results. Using a high-quality DNA source tests whether the two sources have comparable allele numbers, size and frequency, and produce comparable heterozygosity rates. This second check can be used to determine if the DNA amplified from a lower-quality source is particularly prone to allelic dropout.

We suggest that when the DNA is being used to determine the genetic relatedness between individuals, two further checks should be performed: 3) multiple, independent replications of each genotype [Taberlet et al., 1997; Flagstad et al., 1999], and 4) Mendelian checks on known parent-offspring pairs. Performing multiple replications may eventually amplify two alleles in all heterozygous individuals, thereby mitigating the effects of allelic dropout. Finally, Mendelian checks of allele inheritance patterns ensures that all known offspring have at least one inherited parental allele at each locus, further confirming that the STRP primers amplify a single locus.

METHODS

Genotyping Subjects With STRPs on the X Chromosome

The data presented here are part of a larger project to identify paternal siblings among adult female baboons (*Papio cynocephalus*) living in and around Amboseli National Park, Kenya [Hausfater, 1975; Altmann, 1980; Muruthi et al., 1991; Altmann, 1998; Alberts, 1999]. The subjects are 29 adult (having reached menarche) female baboons. Five females were genotyped using blood-derived DNA, while DNA for the remaining 24 females was extracted from feces. DNA from mothers was available for 13 of the 29 adult females but their fathers were not known. Therefore, primers for STRPs on the X chromosome were used to identify paternally-inherited X chromosome haplotypes. The maternally derived allele was determined by identifying the allele that mothers and daughters had in common; the non-maternally derived allele was assumed to be inherited from the father. Because males are haploid on their X chromosome, all daughters inherit the same paternal X chromosome from their father. Thus, paternal half sisters will share their paternally-inherited allele at every X-linked locus, making it much easier to exclude females as paternal half sisters. In contrast, at autosomal loci paternal sisters will share no alleles at a given locus in 25% of the cases. Even in cases in which the mother's DNA was either unavailable or was uninformative (mother's and daughter's genotypes were identical), two females who had no allele in common at a specific locus could be excluded as paternal half sisters at that locus. This would not be true of autosomal loci.

Controls and Validation Checks

Every PCR performed for the study included two types of PCR positive controls. The first was a reaction using high quality human DNA from the Centre d'Études de Polymorphisme Humain (CEPH). The second positive control was a reaction using high-quality DNA derived from baboon blood. Only those PCR reactions that successfully amplified high-quality DNA from both humans (CEPH DNA) and from baboons (blood-derived DNA) were used to calculate the amplification success rates of baboon feces-derived DNA.

In addition to PCR controls, each of the four validation checks that we propose was included in this study to confirm the results. First, the DNA was collected from 29 individually recognized female baboons. Second, the initial screenings of each X-linked primer pair were carried out on blood-derived DNA from five unrelated females. In this way we ensured that these primer pairs amplified polymorphic loci in baboons and yielded consistent results for each individual. Primer pairs that successfully amplified at least three different alleles in the initial screening were then tested on feces-derived DNA from 24 female baboons. Third, multiple replications of each genotype were carried out (see below). Finally, DNA from 10 known mother-daughter pairs (see below) was included to perform Mendelian checks of allele inheritance patterns, ensuring that all offspring had at least one inherited parental allele at each locus.

Sample Collection and DNA Amplification

Fecal samples were collected almost immediately after defecation and stored in 5-ml cryogenic tubes, which were previously filled with 2.5 ml of 95% ethanol. Approximately 2 g of feces were carefully collected, avoiding any contamination with human DNA. The storage tubes were labeled with the donor's name, the

time, and the date, and then sealed with parafilm to prevent leakage. Samples were stored at ambient temperature in the field for up to six months. The fecal samples were frozen at –80°C once they arrived in the U.S. and stored for up to four years before being used for DNA extraction.

Most of the blood samples were collected in 1993. Blood was only collected during specific instances when an animal was darted and anesthetized for other reasons [Sapolsky & Altmann, 1991; Altmann et al., 1996]. Approximately 2–3 ml of blood were collected into vacutainer tubes with EDTA as an anticoagulant. The samples were cooled as soon as possible and then spun down and frozen for shipping within hours of collection [Altmann et al., 1996]. Once in the U.S., blood samples were stored at –80°C for up to five years before DNA was extracted from them.

DNA extracted from blood followed the protocol described in Bruford et al. [1992], and the DNA from feces was extracted using the method described in Taberlet et al. [1997]. The human microsatellite primer pairs were purchased from Research Genetics, Inc. (Huntsville, AL) and were selected because of their location on the X chromosome and because of their relatively high heterozygosity scores in humans of ≥ 0.7.

Each set of 36 PCR reactions was carried out by preparing a single PCR mixture ("cocktail") and delivering 10 μl of this mixture to each of 36 reaction tubes. Reaction tubes contained either 0.5 μl of blood-derived DNA or 1 μl of feces-derived DNA, which had been air-dried. The PCR cocktail was made by adding the following reagents in the following order: 240 μl ultra pure H_20, 36 μl of 10 × Promega™ PCR buffer, 36 μl of 2 mM dNTPs, 36 μl of 25 mM $MgCl_2$ (Promega), 2.9 μl of 25 pMol forward and reverse primers, 2 μl of alpha ^{32}P-dCTP, and 5 μl of Promega™ *Taq* polymerase (Promega). Ten μl from the resulting 360.8 μl cocktail were then divided among 36 PCR reactions. The final concentrations were as follows: 1 × Promega™ PCR buffer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.16 μM forward and reverse primers, 100 pMol ^{32}P-dCTP, and 0.7 units Promega™ *Taq* polymerase.

All amplifications were carried out under the following PCR conditions using a Perkin Elmer GeneAmp 9600 thermal cycler: samples were denatured for 5 min at 94°C followed by 40 cycles of denaturing for 30 sec at 94°C, annealing for 75 sec at 54°C, and extending for 30 sec at 72°C. Following a final extension period for 10 min at 72°C, the samples were held at 6°C.

PCR products were resolved on a denaturing polyacrylamide gel (6.7%) and were visualized on a Storm phosphorous screen using Image Quant™ software.

Multiple Replications of Each Genotype for Feces-Derived DNA

The feces-derived DNA used to genotype each female was taken from between two and six fecal samples (collected on different days), and from between three and 10 independent extractions. Those extractions that amplified DNA consistently were used preferentially to those that did not. Because of potential allelic dropout, each female's DNA was initially amplified at least 8 to 12 times at each of the five loci, and genotype assignment was based on a consensus of alleles from these multiple amplifications [see Taberlet et al., 1997]. If, during the first 8–12 replications, two alleles were amplified unambiguously at least three times, the individual was considered heterozygous and the genotyping at that locus was considered resolved. The two alleles did not necessarily have to appear in the same amplification product. That is, individuals who appeared "homozygous" for two different alleles were considered as heterozygotes, even if the two alleles never appeared in the same amplification [Taberlet et al., 1997]. Indi-

viduals were considered homozygous if only one allele amplified after 16 replication attempts.

Mendelian Checks

Maternity was based on 1) observations of the external signs of ovulation dates and pregnancy whereby birth due-dates are determined; 2) observations of females who display postpartum signs such as blood on the perineum or hands, along with the presence of an infant who is only hours to days old; or 3) observations, in rare cases, of the actual birth. Maternity in baboons is unambiguous. Baboons are not seasonal breeders, so females rarely give birth very close in time (the only circumstance under which one female could successfully adopt another's infant). Further, allomaternal care is uncommon, and adoption has never occurred in Amboseli.

RESULTS

Less than half (12/29, 41%) of the primers successfully amplified blood-derived baboon DNA (Table I). Of the 12 primers that amplified DNA from baboon blood, six revealed only a single band in all individuals, suggesting that these loci are monomorphic in baboons. The six remaining primers amplified baboon DNA that was polymorphic, i.e., they yielded three or more alleles among the five test animals. These six primers were then used on DNA extracted from feces and again successfully amplified polymorphic DNA. One primer (GATA48H04) passed the first three utility tests, but was rejected for further use as it did not pass the Mendelian check: three of the six mother-daughter pairs tested had no shared alleles (Table II). In two cases, both mother and daughter were heterozygous for different alleles, so the mismatch could not be attributed to allelic dropout. In the third case, the mother was heterozygous and the daughter was homozygous, and neither maternal band was present. This mismatch was not likely to be due to allelic dropout as the homozygous genotype was confirmed after nine replications using high-quality, blood-derived DNA (Table II).

The remaining five human STRP primers were considered suitable for genotyping from both blood- and feces-derived DNA, and were used in subsequent relatedness studies. These five STRPs (AFM240WA9, GATA69D06, GATA124B04, GATA144D04, and GATA164D10) were all polymorphic, each having between six and eight alleles (Table III). Importantly, they each passed all 58 possible Mendelian checks, i.e., all 58 mother-daughter pairs had at least one shared allele. However, they varied in their ability to amplify feces-derived DNA, ranging from 44 % to 67% overall success rates (Table III).

Not surprisingly, DNA extracted from blood had a higher success rate than did feces-derived DNA: 97% (34/35) of independent amplifications of blood-derived DNA compared with 53% (87/163) of independent amplifications of feces-derived DNA were successful (n = 198, Pearson's χ^2, $P < .0001$). Furthermore, feces- but not blood-derived DNA was subject to allelic drop-out. No cases of allelic dropout were observed in 35 independent blood-derived DNA amplifications, whereas DNA extracted from feces exhibited allelic dropout in 48% (66/137) of independent PCR reactions (total n = 172, Pearson's χ^2, $P < 0.0001$). After multiple replications of each genotype, heterozygosity levels were eventually indistinguishable between blood- and feces-derived DNA; blood-derived DNA produced 89% (31/35) independent heterozygous genotypes, while feces-derived DNA produced 86% (97/113) independent heterozygous genotypes (Pearson's χ^2, $P = 0.67$).

TABLE I. Human Microsatellite Primer Pairs Tested on Baboon DNA*

Primers that failed to amplify baboon DNA derived from blood, but did amplify human DNA	Primers that amplified baboon DNA but <10% of test animals were heterozygous	Primer amplified baboon DNA and was polymorphic but failed Mendelian checks	Primers that amplified baboon DNA, were ≥79% heterozygous in test animals, and passed Mendelian checks
AMF203YD8	AFM317YE9	GATA48H04	GATA124B04
GATA10C11	DXS1227		GATA144D04
GATA124E07	DXS207		GATA165D10
DATA151F04	DXS6799		GATA69D06
GATA160B08	GATA125B12		AFM240WA9
GATA172D05	AFM248WF9		
GATA186D06			
GATA192D07			
GATA28C05			
GATA31B12			
GATA31F01			
GATA42G01			
GATA72E05			
GATA87A12			
GATA64D08			
GGAT3F08			
XS1059			

*The primers in the first column were tested on DNA extracted from baboon blood (n = 5 individuals), and on high-quality human DNA (CEPH, n = 2 individuals, 1 male and 1 female) included as a positive PCR control and to insure that the male had only one allele at each X chromosome locus tested. The primers in the next three columns successfully amplified DNA extracted from both baboon blood (n = 5, same individuals used in the initial tests) and feces (n ≤ 24 individuals, depending on whether the primer passed all checks and was used to genotype all individuals in the study). Information on primer sequences, allele sizes, and heterozygosities in human can be obtained at www.marshmed.org

TABLE II. GATA48H04 Fails Mendelian Check[†]

		DNA source	Allele 1 ()	Allele 2 ()	No. of tissue samples	No. of extractions	No. of PCRs
Mom 1		feces	1 (6)	5 (6)	2	3	1
	Daughter 1*	blood	3 (8)	6 (8)	1	2	4
	Daughter 2	feces	1 (8)	5 (8)	1	1	1
Mom 2		feces	3 (12)	8 (12)	2	3	2
	Daughter 1	feces	3 (21)	NA	2	3	2
	Daughter 2*	blood	6 (15)	NA	1	2	6
	Daughter 3	feces	3 (7)	NA	2	2	1
	Daughter 4*	feces	5 (7)	6 (3)	2	2	1

[†]The number of replications in which the allele amplified is in the (). Daughters with asterisks are those with no shared alleles with their mothers. NA (not applicable) refers to homozygous individuals with no second, differently-sized allele. Number of tissue samples represents the number of blood or fecal samples collected on different days. Notice blood was only collected on one day for the two females for whom blood was available, while most fecally-derived genotypes were based on more than one fecal sample. Number of extractions refers to the number of independent extractions from one or more fecal samples. Number of PCRs refers to the number of independent PCR amplifications used to generate the number of replications noted inside (). Therefore, all of Mom 1's six genotypes were generated from three extractions of 2 fecal samples amplified in one PCR reaction.

DISCUSSION

Ensuring accuracy of genotyping results is especially important when using cross-species amplification and/or low-quality DNA, and is even more critical when using the genotypes to determine relatedness between individuals. The success rate of cross-species amplifications that were informative in this study agrees closely with that of others: Launhardt et al. [1998] found that only five of the 32 (16%) human primer pairs amplified polymorphic DNA extracted from langur feces, while Coote and Bruford [1996] found that 15 out of 85 (18%) human primers amplified polymorphic baboon DNA. Furthermore, the number of alleles and allelic frequencies resulting from our studies are comparable with those reported

TABLE III. X-Linked STRPs*

STRP	cM from p-terminus[a]	No. of alleles in baboons	% amplification success	% allelic dropout	% heterozygous genotypes after multiple replicates
GATA164D10	4.39	5	47	69	97
GATA124B04	8.76	7	55	33	79
AFM240WA9	52.63	6	67	23	87
GATA144D04	71.29	8	52	37	87
GATA69D06	93.17	6	44	47	83
Average		6.4	53	42	87

*Results given are based on DNA extracted from baboon feces (n = 24 individuals genotyped at 5 loci for a total of 110 genotypes). All PCR reactions included high-quality human DNA (CEPH) used as a PCR positive control and blood-derived baboon DNA as a species specific PCR positive control. Percent amplification success was calculated by dividing the number of replicates that amplified by the total number of replicates included in which the high-quality DNA from humans and baboons amplified. Percent allelic dropout was calculated by dividing the percent of homozygous genotypes from feces-derived genotypes by the percent of homozygous genotypes generated from blood-derived baboon DNA. Percent of heterozygous genotypes was calculated simply by dividing the number of heterozygous genotypes by the total number of genotypes. Number and size of alleles in humans can be obtained from www. marshmed.org

[a]Based on human chromosome map from http://www.marshmed.org

by others using feces-derived DNA [Reed et al., 1997; Launhardt et al., 1998; Flagstad et al., 1999].

The results presented here corroborate those reported by others. We are aware of only one previous study, however, that utilizes the known relationships between animals to ensure specific amplification of single loci [Launhardt et al., 1998]. We particularly highlight here the importance of multiple replications and of Mendelian checks when using cross-species amplification of feces-derived DNA by demonstrating nonspecific amplification of an apparently polymorphic and replicable STRP primer. In this study, STRP primer GATA48H04 passed the traditional checks during the screening process. It amplified baboon DNA extracted from the blood and feces of known individuals, and showed consistent, polymorphic genotypes in repeated replications. However, three of the six mother-daughter pairs tested had no shared alleles at this locus. These mother-daughter pairs exhibited Mendelian inheritance at every other locus. This suggests that mislabeling of samples in the field was unlikely to be the cause of this problem. Furthermore, two of the three daughters who mismatched with their mothers were genotyped at the mismatched locus on between three and four gels, in three independent PCRs, with consistent results. This suggests that sample mix-ups in the lab were an unlikely source of the problem; the sample mix-up would have had to happen for both females three times independently, all with the GATA48H04 primer pair. Banding patterns that do not follow Mendelian segregating patterns may result from non-specific amplification of a second locus (amplification of a non-target locus), or from polymorphisms in the primer binding sites. Some primers such as GATA48H04 may nonspecifically amplify two loci in the genome, with the mother's DNA amplified at one locus and the daughter's DNA amplified at a second locus.

Although samples of unrelated animals may be sufficient for identifying polymorphic STRP loci in many population-based studies, investigations of relatedness between individuals or social groups require additional checks that ensure the specificity of the primers. Our data indicate that 17% (1 of 6) of polymorphic loci that were amplified in baboons using human primers resulted in non-Mendelian segregation patterns in mother-daughter pairs, despite the fact that no one individual had ≥ 2 bands. The use of primer pairs that are nonspecific could lead to misidentification of relatives and underestimates of relatedness.

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