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Microsatellite scoring errors associated with noninvasive genotyping based on nuclear DNA amplified from shed hair

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Abstract

In the context of a study of wild chimpanzees, *Pan troglodytes verus*, we found that genotypes based on single PCR amplifications of microsatellite loci from single shed hair have a high error rate. We quantified error rates using the comparable results of 791 single shed hair PCR amplifications of 11 microsatellite loci of 18 known individuals. The most frequent error was the amplification of only one of the two alleles present at a heterozygous locus. This phenomenon, called allelic dropout, produced false homozygotes in 31% of single-hair amplifications. There was no difference in the probability of preferential amplification between longer and shorter alleles. The probability of scoring false homozygotes can be reduced to below 0.05 by three separate amplifications from single hairs of the same individual or by pooling hair samples from the same individual. In this study an additional 5.6% of the amplifications gave wrong genotypes because of contamination, labelling and loading errors, and possibly amplification artefacts. In contrast, amplifications from plucked hair taken from four dead individuals gave consistent results (error rate < 0.01%, n = 120). Allelic dropout becomes a problem when the DNA concentration falls below 0.05 ng/10 μL in the template as it can with shed hair, and extracts from faeces and masticated plant matter.

Keywords: chimpanzees, microsatellite, PCR, amplification, hair, null alleles, allelic dropout, *Pan*

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Introduction

The discovery that nuclear and mitochondrial DNA can be amplified from single shed (telogen) hairs promises to revolutionize the study of genetic aspects of some mammal populations (Woodruff 1993; Morin & Woodruff 1996). The technique was first described by Higuchi *et al.* (1988) and has been widely applied to humans by forensic scientists (e.g. Uchihi *et al.* 1992, Ruano *et al.* 1992) and first applied to other mammals by Takasaki & Takenaka (1991). It allows noninvasive genotyping of individuals from wild mammal populations and eliminates the need for biopsy. Among the mammals that have been studied using hair-derived DNA are chimpanzees (Morin & Woodruff 1992; Morin *et al.* 1994a, b), European brown bear (Taberlet *et al.* 1993; Taberlet & Bouvet 1994), gibbons (Garza & Woodruff 1992), wombats (Taylor 1995) and gorillas (Garner & Ryder 1996).

In spite of its promise and the fact that DNA is easily obtained from single shed hair, genotyping from hair requires caution. Several authors have already described problems associated with PCR amplification of trace amounts of DNA. Preferential amplification, also called allelic dropout, has been observed in several studies (Walsh *et al.* 1992; Navidi *et al.* 1992; Gerloff *et al.* 1995, Taberlet *et al.* 1996). The minute quantity (subnanogram) of nuclear DNA found in single shed hair extracts can cause allelic dropout due to stochastic amplification of only one of the two alleles present in the template. Low template quantity (less than 30 cells) has also been associated with amplification artefacts in human $(CA)_n$ repeat polymorphisms, i.e. the preferential amplification of artefactual alleles due to slippage of the polymerase during the PCR (Foucault *et al.* 1996). Other factors such as mutations in the flanking primer sequences can also lead to

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nonamplifying or 'null-' alleles (Callen *et al.* 1993; Pemberton *et al.* 1995; Paetkau & Strobeck 1995) but they are not caused by low template quantity and are not discussed here. In this paper we document the magnitude of allelic dropout using results from the first author's on-going study of the population genetics of West African chimpanzees, *Pan troglodytes verus*. Although our investigation has an intrinsic *post hoc* limitation, our observations point to the significant nature of this phenomenon and suggest ways others can take to minimize genotyping errors.

Materials and methods

Tissue collection

Multiple shed hair samples were available for 55 chimpanzees in an habituated community in the Taï forest of Côte d'Ivoire, West Africa (Boesch & Boesch 1989, 1990). The animals are all individually known and adults were observed making new nests in the forest canopy, sleeping in them alone, and leaving in the morning of the next day before the first author climbed to the nest to collect the hair. Hairs were picked up wearing latex gloves to avoid human contamination. Each hair was placed individually in a paper envelope and kept at ambient temperature in a dry box containing regularly desiccated silica gel for up to 6 months prior to DNA extraction in the laboratory. For the majority of individuals we collected hair from more than one nest. No tissue or blood samples were available from any of the individuals of the study community, as obtaining such samples would require darting animals and probably destroying the community's behavioural habituation. However, we were able to pluck hair directly from four additional chimpanzees (two known and two unknown individuals) whose corpses were discovered in the forest within 24 h of death.

DNA extraction from shed (telogen) hair

Following current standard methodology, two extraction methods were used. The first is an extraction in 200 µL 5% Chelex-100 (BioRad) suspension: the hair is first washed with 70% ethanol and de-ionized distilled water and then 3–5 mm of the proximal end with the hair follicle is cut off and allowed to drop into the Chelex solution. All handling is carried out in a closed UV isolation cabinet wearing a face mask and using sterile forceps and scissors that are washed in bleach, and rinsed in alcohol and (DI) water between each sample treatment. The solution is then heated at 56 °C for 2 h, vortexed at high speed for 10 s, put in boiling water for 10 min, vortexed again for 10 s and finally centrifuged for 3 min at 16 000 *g*. The extracts are stored in the dark at 4 °C.

The second method of extraction involves a digestion of the cut off, proximal 3–5 mm portion of the hair with protease K overnight at 56 °C in a hair lysis buffer (10 nM Tris pH 8.0, 50 mg/mL Protease K, 35 mM DTT, 0.9% Laureth 10), before Chelex is added (C. Orrego, personal communication). Subsequent procedures follow the outline above.

Extractions were all made from one hair at a time. Sample preparation and PCR set-up were performed in two different closed UV hoods. Each batch of DNA extractions was controlled for contamination by amplifying at least two Chelex blanks.

DNA extraction from other sources

We also extracted DNA from buccal cells in 'wadges'. These are pieces of masticated fruit pulp left behind by identified chimpanzees we had observed feeding. The olive-sized pulp samples were collected in 40 mL of 70% ethanol and transported at room temperature. The ethanol was filtered using autoclaved cheese cloth and centrifuged at low speed for 10 min. The supernatant was discarded and the pellet was washed twice with DI water. Part of the pellet (≈ 0.2 g) was then used in a silica membrane based QIAamp® tissue-extraction kit (QIAGENE). These additional samples allowed us to verify the genotypes of several individuals as well as to verify the hair-based genotypes of mother and offspring collected in shared nests. Results from these samples were not included in the analysis of error frequency.

Microsatellites studied

Heterologous human primers were used to amplify 11 chimpanzee microsatellite loci: dinucleotide repeats, *Mfd3*, *Mfd18*, *Mfd23*, *Mfd32* and *LL1* (Weber & May 1989; 1990; Weber *et al.* 1990; Litt & Luty 1989), trinucleotide repeats, *Humfabp* and *HumPla2a* (Edwards *et al.* 1991) and tetranucleotide repeats, *Mbp2*, *vWF*, *Rena4*, and *Fesps* (Kimpton *et al.* 1992; Polymeropoulos *et al.* 1992a,b). All have been described as perfect di-, tri- and tetranucleotide repeats in humans.

PCR conditions

PCR reactions were run in a 20 µL volume in HYBAID Thermal Reactors. One unit of Amplitaq® (Perkin Elmer Cetus) per reaction was added to 1.1 µL diluted Taqstart® antibody (CLONTECH). After 5 min it was added to 10 µL of 10 nM Tris (pH 8.3), 0.01% gelatin, 1.5 mM $MgCl_2$, 0.2 mM dA, G, C, T, 100 mM KCl, 10 pmol of unlabelled primer (+), 10 pmol of unlabeled primer (–), and 2 pmol of end-labelled (γ-^{32}P, 3000 Ci/mmol). Finally, 10 µL of template solution (0.05–0.1 ng of DNA; see quantification, below) is added followed by two drops of mineral oil to prevent evaporation. The antibody immobilizes the enzyme until

the first denaturing cycle, reducing the occurrence of non-specific reactions during reaction set up. After an initial soaking at 94 °C for 3 min the reactions were run through 35 cycles of denaturing at 92 °C for 60 s followed by annealing at 55 °C for 60 s and extension at 74 °C for 30 s. Dinucleotide repeats were amplified in a compound reaction with 3 min of soaking at 93 °C, seven cycles of denaturing at 92 °C for 30 s, annealing at 55 °C for 60 s and extension at 72 °C for 90 s, followed by a set of 30 cycles with denaturing at 89 °C for 30 s, annealing at 54 °C for 60 s and extension at 72 °C for 90 s. A 4-μL aliquot of the PCR product plus loading dye was run on 6% denaturing polyacrylamide gels (Long-Ranger®; AT Biochem) at 2000 V and 500 mA for 2–4 h depending on the size of the product. Gels were bound to filter paper and dried in a gel drier before being exposed to radiography films (Kodak Biomax®) overnight. A sequence of M13 (using the – 40 sequencing primer from the US Biochemical Sequenase® kit) was run on each gel as a size standard. Alleles were scored by eye by comparing the position of the bands to the M13 ladder. All reactions were run with a negative control (water) and a positive control consisting of a single hair plucked from the first author's arm. The product from the positive control was found to be highly consistent and was used as a size standard at a later stage. Four of the 11 loci used have nonoverlapping size ranges for known human and chimpanzee alleles, allowing for the detection of human contamination. PCR amplifications were repeated multiple times with aliquots from the same single hair extraction as well as with aliquots from different single hair extracts from the same individuals. Navidi *et al.* (1992) proposed a multiple-tubes approach for accurate genotyping of very small DNA samples. Our sequential amplification of multiple 10 μL aliquots from 200 μL Chelex extracts is a similar approach. However, because we usually face limited sample quantities, we adapted the number of repeated amplifications to the type of result obtained (see below).

DNA quantification

Chelex extractions were measured for DNA content by fluorometry. Aliquots of 50 μL at various concentrations were mixed with 50 μL of Pico Green® dye (Molecular Probes), left to incubate at room temperature for 10 min and were measured on a fluorescence multiwell plate reader (Perseptive Biosystems) at a gain of 95%. The standard curve for the measures was created using a dilution series of human placental DNA containing 2.5, 10, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200 ng/mL.

Samples included in the analysis

Out of the 55 members of the Taï community genotyped only 18 individuals known to have slept alone in their nests were included in this analysis, i.e. adult males, childless females and juveniles. This avoids the further complication caused by the potential presence of two different genotypes in mother–infant nests. From these 18 individuals, we considered only amplifications that yielded detectable product. For a total of 1090 such amplifications on 176 genotypes the amplification products were sorted into apparently homozygote genotypes (48 genotypes, 299 amplifications) and apparently heterozygote genotypes (128 genotypes, 791 amplifications). For the apparently homozygote genotypes, amplifications were repeated more than three times (mean = 6.1). The minimum criterion for accepting a heterozygous genotype was the following: it must have appeared in two or more independent amplifications using two different hairs from the same individuals or in one amplification after each of the two alleles had appeared as homozygous genotypes in two or more prior amplifications. Only the amplifications for genotypes at which these 18 individuals were heterozygous (791 amplifications) were used to estimate the frequency of allelic dropout.

Results

Amplification and genotyping was successful from shed hair up to 5 years after collection. The two extraction methods used for shed hair and the method used for buccal cells yielded similar results and there was no apparent difference in the quality of the DNA extractions. Approximately 50% of all shed hair extracts using either method gave amplification products in at least some of the amplifications.

The mean DNA concentration for single shed hair was 0.0174 ng/mL ± 0.015, corresponding to a total mean yield of 3.6 ng per single shed hair. Concentration varied strongly between extracts. The good-quality extracts, those consistently yielding two alleles for heterozygous loci, contained > 6 ng. Ten-microlitre aliquots of extractions containing as little as 2 ng (template quantity = 0.2 ng/reaction) regularly gave amplification products but did so much less consistently. Samples measured at less than 0.01 ng/mL rarely produced any amplification product. Ten Chelex blanks, tested multiple times and giving no amplification product, were measured and had a mean value of 0.007 ng/mL. We take this as an indication that the measures carried out using such small amounts of DNA and such a high gain in the fluorescence reader are only of limited precision, but note that our results are comparable with those reported by Ruano *et al.* (1992) who found an average yield of 1 ng per shed hair for humans. Several attempts at concentrating the Chelex extracts from shed hair failed to improve the amplification result and would usually allow only one or two amplifications.

We determined the critical DNA concentration at which one starts to observe allelic dropout by testing four

Table 1 Combined single shed hair PCR results for each of the 18 individual chimpanzees, out of 11 MSAT loci only those at which the individuals were hetrozygous are considered. Error rates vary between individuals for unknown reasons. mean = 0.31 ± 0.0425E, χ^2 test, d.f. = $P < .001$.

		Individuals*																	
Result type		AGA	ALI	BEL	BRA	BRU	DAR	FIT	GIP	GIT	GOY	KEN	MAC	MAR	OND	ROU	ULY	XER	ZER	Total
Number of heterozygous loci		6	10	7	3	6	7	9	9	8	6	7	7	9	6	9	7	6	5	
Correct		27	45	24	10	42	42	35	23	31	20	31	35	11	19	39	28	17	20	499
Error type 2	Shorter allele missing	6	11	2	0	13	6	1	7	7	5	13	11	7	0	7	3	8	2	109
	Longer allele missing	6	14	3	0	12	11	6	5	6	9	12	7	26	3	6	6	7	0	139
Error type 1	Wrong genotype	0	8	0	0	5	2	3	3	2	2	1	1	8	1	4	3	1	0	44
	Total	39	78	29	10	72	61	45	38	46	36	57	54	52	23	56	40	33	22	791

dilution series made from two good-quality plucked hair extractions from two wild individuals measured at 0.19 ng/mL and 0.12 ng/mL and two DNA extractions from blood of two captive chimpanzees (*verus* subspecies, Phillip Morin kindly provided the DNA) with genomic DNA (20 ng/mL). We amplified a locus for which all four individuals were known to be heterozygous. For all four samples the concentration at which we started to observe allelic dropout was 0.005 ng/mL or a total template quantity of 0.05 ng of DNA in 10 µL. We conclude that when amplifying nuclear DNA from shed hair, the total template quantity will frequently be 1 ng per hair or less (200 µL of 0.005 ng/mL = 1 ng). When doing multiple amplifications from such extractions allelic dropout is to be expected.

In contrast, the extracts from plucked chimpanzee hairs gave a yield of more than 20 ng (two independent measurements, 27 extracts from four individuals). This yield is comparable with the one reported for human plucked hair by Uchihi *et al.* 1992).

Measure of replicability and definition of error

We defined error as two or more nonidentical PCR results at a locus. Two types of errors were observed using shed hair.

Error type 1: wrong genotype. 5.6% of the shed hair PCR amplifications (15% of errors), gave a completely wrong genotype as concluded after further amplifications using the same as well as other hair extracts from the same individual.

Error type 2: false homozygote. 31.3% of the shed hair PCR amplifications (85% of the errors) gave 'false homozygotes' for which one of the two alleles of the real genotype was not amplified due to allelic dropout.

Pooling both error types, for all individuals over all 11 loci, the mean error rate for single locus genotypes from shed hair was 37%. Amplifications of DNA extracted from plucked hair samples from the four dead chimpanzees gave consistently replicable results, i.e. identical genotype scored 3–4 times at each locus for each individual (total error rate < 1%, $n = 120$). Extractions from the first author's plucked arm hairs also gave identical results in more than 1000 amplifications. For the shed hair, the results varied from hair to hair for the same individual and between individuals. We interpret this variability as caused by the quality and number of follicular cells attached to each hair root and the state of their preservation.

The 18 individuals differed in the frequency of giving wrong genotypes (Table 1), but there was no difference in error rates among the 11 loci (see Table 2). In cases with allelic dropout there was no difference between the

Table 2 Single shed hair PCR results for heterozygous loci of 18 combined individuals at 11 MSAT loci. there is no significant difference in error rate between loci (ANOVA, using fraction of errors per individuals as replicates d.f. = 10, $F = 1.34$, $P = 0.215$). There is no significant difference in error rate between the longer and shorter alleles. (paired t-test, d.f. = 10, $t = 2.16$, $P > 0.05$). There is no significant difference in type 1 error rate between CA repeats and tri-/tetranucleotide repeats (umnpaired t-test, d.f. = 9, $t = 1.01$, $P = 0.338$)

		Loci											
Result type	Individuals	Fabp 10	Pla2a 11	Mfd3 13	Mfd18 12	Mfd23 15	Mfd32 13	Mbp2 11	vWF 16	Rena4 8	Fesps 6	LL1 13	Total
Correct		52	69	38	56	63	46	46	64	16	17	32	499
Error type 2	Shorter allele missing	12	11	13	3	8	6	14	18	7	4	13	109
	Longer allele missing	15	10	17	18	17	13	14	14	2	3	16	139
Error type 1	Wrong genotype	5	3	1	6	3	6	7	6	0	0	7	44
	Total	84	93	69	83	91	71	81	102	25	24	68	791

probability of the shorter or longer allele not being amplified (Table 2). We were unable to compare the error rate observed in this study to that found in other such studies as error rates are not reported by most authors.

Discussion

Errors of the first type can only be detected by repeating amplifications for each sample. Between two and five amplifications with different hair extracts are needed to verify the genotypes and rule out error sources like contamination, amplification artefact (for $(CA)_n$ repeats see below) switching of sample, loading, labelling or gel scoring error. If amplification artefact were responsible for a large part of the type 1 errors, we would expect a difference in the frequency of type 1 error between the dinucleotide $(CA)_n$ repeats and the trinucleotide and tetranucleotide repeats, given that trinucleotides have been shown to be much less prone to slippage artefacts (Zhang *et al.* 1994; Foucault *et al.* 1996). No such difference was observed indicating that preferential amplification of artefactual bands did not contribute substantially to the observed cases of type 1 errors. When it becomes clear after three amplifications, that there is a type 1 error in the results, obviously, more amplifications will have to be performed and if possible using new extracts from new samples. We agree with Taberlet *et al.* (1996) that the required number of individual amplifications will depend on the results of an initial number of (3–7) amplifications with clear product, given that sample quantity is expected to be limited in this type of study. We have to keep in mind that if contamination occurs during sample collection or extraction, different samples could be contaminated by the same source and would thus not represent independent controls. In our case, all samples were collected and all extractions were performed by the senior author, meaning that we have a direct control for the most likely source of contamination.

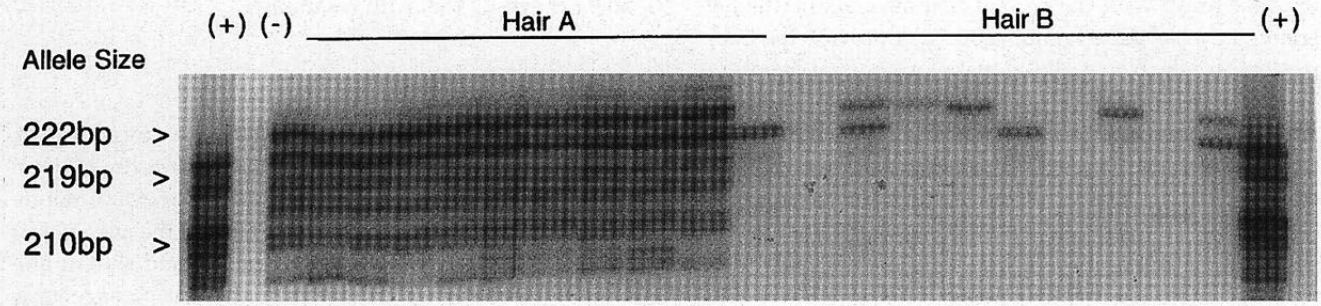

Fig. 1 Twenty independent PCR results using aliquots from two different shed hair extracts of the male chimpanzee, *Brutus*. The locus is Humfabp, a trinucleotide repeat. The first and last lane show size standards (genotype of senior author), the second shows a negative control. In lane 3–12, all 10 aliquots from hair A yield heterozygote genotypes, in lanes 13–24, 10 aliquots from hair B yield four different results: no band, one band the size of the shorter allele, one band the size of the longer allele, and both bands. Only the latter is the correct genotype. This illustrates the potential difference in template quality between two single shed hair samples from the same animal.

For the much more frequent problem of allelic dropout, our results suggest the following strategy: one can estimate the number of repeated amplifications required in order to lower the probability of such errors to some acceptable value. Here we do this by neglecting the 5.6% error rate for wrong genotypes, and considering only the much larger probability of obtaining a false homozygote. Considering the rate of allelic dropout error as a lower boundary of error probability, we can calculate the probability of the same allele appearing alone (without the other allele appearing) in n consecutive amplifications. In other words we are asking: after how many reactions yielding the same and only that allele can we be confident that the genotype is really homozygous and that we are not misclassifying a heterozygote as a homozygote?

Assuming that we identified most of the truly heterozygous loci (values for expected heterozygosity are high at most microsatellite loci) we can use the error rate observed in those cases, i.e. all PCR amplifications carried on heterozygous template, to estimate how many heterozygotes we have missed in the total sample. Those would be cases in which the same allele dropped out in six independent amplifications using different hair extractions as template. In our case 299 PCR of 48 genotypes classified as homozygotes gives an estimated error rate of $0.31^6 = 0.00089$ or less than one genotype. We are admittedly defining 'correct' a posteriori and there will be a residual number of cases for which we misclassified a genotype as homozygote even after six independent amplifications using different hair extractions. This number is likely to be very small however. By their very nature field studies of this type cannot provide us with alternative sources of DNA for every individual studied and we have no other means for evaluating the importance of that residual error. This could however, be determined in a rigorous laboratory study.

Once we obtained one of the two possible false homozygotes, we have a 94% probability of knowing one of the two real alleles. If the real genotype is heterozygote, the first result with the second real allele, either as heterozygote or as 'false homozygote' will provide the information on which two alleles make up the real genotype. Thus we can use the sequential probabilities of partial amplification errors to calculate the critical numbers of amplifications needed to reach a certain level of confidence:

$$P(\text{false homozygote}) = (K) \times (K/2)^{n-1}$$

where K is the observed frequency of false homozygotes averaged over all individuals and loci (error type 2, in our case = 0.313) and n is the number of repeated amplifications. In our case, three separate amplifications reduce the probability of missing the same of the two alleles to < 0.05. Only one out of the 18 individuals considered had an error rate requiring more than three separate amplifications to yield a value below 0.05.

Pooling of several hairs from a single night nest does increase the quantity of template DNA, but also creates the risk of erroneous genotypes if it is not proven that all the hairs are from the same individual. Furthermore, inhibition of PCR reactions by melanin from the hair shaft has been demonstrated by Uchihi *et al.* 1992). For mother–offspring pairs, that in the case of chimpanzees share their nests for the first 4–6 years of the infant's life, one has no choice but to work with one hair at a time in order to tell mother and offspring genotypes apart. The same problem may arise when using hair from other species found on abrasive surfaces (e.g. rocks, tree bark or barbed wire) as one cannot exclude the possibility that several animals scratched themselves at the same site.

Among genetic studies of natural animal populations very few are supported by abundant observations on behavioural interactions and social structure. In fact, noninvasive genotyping methods are increasingly used as a way of inferring relationships and social structure based on observed genetic structure. We are in the fortunate position of knowing relationships (mother–offspring) in the habituated chimpanzee community of the Taï Forest. This allows us to judge the validity of the genotypes that we obtained. Any results in which known mother–offspring pairs do not share at least one allele at a particular locus can be rejected as false and due to either contamination or allelic dropout. This type of control is no guarantee, however, for the detection of all partial amplifications. Despite such problems noninvasive genotyping can yield replicable results as illustrated by the following facts in our study: first all mother–offspring genotypes at Taï verified using independent wadge samples ($n = 8$) showed a shared allele at each of the 11 loci. Second the same multilocus genotype from hair samples collected in different nests was obtained for all individuals with multiple nests sampled ($n = 15$). Finally, one individual (subadult male) sampled only as bone, shared at least one microsatellite allele at every locus with his mother.

Conclusion

Our observations show that where circumstances permit, rather than collecting shed hair, obtaining freshly plucked hair is clearly worth the additional effort. Several noninvasive methods await full development: the use of soft sticky projectiles attached to fishing line and shot at animals or the use of Velcro-like material near passageways, trails or den-entries. If however, shed hair is the only option, [homozygous] genotypes should not be accepted before PCR amplifications have been repeated with extracts from two or more hairs at least three times. If one can be confident about several hairs being from the same

individuals, pooling 3–10 hairs into the same DNA extract while keeping the quantity of hair shafts as low as possible (to avoid inhibition) will also reduce the problem of allelic dropout.

In our experience, this allelic dropout problem appears to be specific to single shed hairs and buccal cells extracted from chewed fruit pulp. In this laboratory it has not been observed in studies using larger DNA sources including mammalian ear and toe clips in ethanol, dry bird feathers, and reptile blood in ethanol. Researchers studying wild animal populations using shed hair, buccal cells from chewed plant material, and fecal samples must be aware of the high error rate associated with the amplification from such trace amount DNA samples. They must keep in mind the limitations of this method when planning their sample collection and the number of amplifications to be subsequently performed on each sample.

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References

Boesch C, Boesch H (1989) Hunting behaviour of wild chimpanzees in the Taï National Park. *American Journal of Physial Anthropology* **78**, 547–573.

Boesch C Boesch H 1990 Tool use and tool making in wild chimpanzees. *Folia Primatologica*, **54**, 86–99.

Callen DF, Thompson AD, Shen Y, Phillips H *et al.* (1993) Incidence and origin of 'null' alleles in the (AC) n microsatellite markers. *American Journal of Human Genetics*, **52**, 922–927.

Edwards A, Civitello A, Hammond HA, Caskey CT (1991) DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. *American Journal of Human Genetics*, **49**, 746–756.

Foucault F, Praz F, Jaulin C, Amor-Gueret M (1996) Experimental limits of PCR analysis of $(CA)_n$ repeat alterations. *Trends in Genetics*, **12**, 450–452.

Garner KJ, Ryder OA (1996) Mitochondrial DNA diversity in gorillas. *Molecular Phylogenetics and Evolution*, **6**, 39–48.

Garza JC, Woodruff DS (1992) A Phylogenetic study of the gibbons (*Hylobates*) using DNA obtained non-invasively from hair. *Molecular Phylogenetics and Evolution*, **1**, 202–210.

Gerloff U, Sclötterer C, Rassmann K, *et al.* (1995) Amplification of hypervariable simple sequence repeats (microsatellites) from excremental DNA of wild bonobos (*Pan paniscus*). *Molecular Ecology*, **4**, 515–518.

Higuchi R, von Beroldingen CH, Sensabaugh GF, Erlich HA (1988) DNA typing from single hairs. *Nature*, **332**, 543–546.

Kimpton C, Walton A, Gill P (1992) A further tetranucleotide repeat polymorphism in the vWF gene. *Human Molecular Genetics*, **1**, 287.

Litt M, Luty JA (1989) A hypervariable microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within the cardiac actin gene. *American Journal of Human Genetics* **44**, 397–401.

Morin PA, Moore JJ, Chakraborty R *et al.* (1994a) Kin selection, social structure, gene flow, and the evolution of chimpanzees. *Science*, **265**, 1193–1201.

Morin PA, Wallis J, Moore JJ, Woodruff DS (1994b) Paternity exclusion in a community of wild chimpanzees. *Molecular Ecology*, **3**, 469–478.

Morin PA, Woodruff DS (1992) Paternity exclusion using multiple hypervariable microsatellite loci amplified from nuclear DNA of hair cells. In: *Paternity in Primates: Genetic Tests and Theories* (eds Martin RD, Dixon AF, Wickings EJ), pp. 63–81. Karger, Basel.

Morin PA, Woodruff DS (1996) Non-invasive genotyping for vertebrate conservation. In: *Molecular Genetic Approaches to Conservation* (eds Smith TB, Wayne RK), pp. 298–313, Oxford University Press, Oxford.

Navidi W, Arnheim N, Waterman MS (1992) A multiple-tubes approach for accurate genotyping of very small DNA samples by using PCR: statistical considerations. *American Journal of Human Genetics*, **50**, 347–359.

Paetkau D, Strobeck C (1995) The molecular basis and evolutionary history of a microsatellite null allele in bears. *Molecular Ecology*, **4**, 519–520.

Pemberton JM, Slate J, Bancroft DR, Barrett JA (1995) Non-amplifying alleles at microsatellite loci: a caution for parentage and population studies. *Molecular Ecology*, **4**, 249–252.

Polymeropoulos MH, Xiao H, Merril CR (1992a) Tetranucleotide repeat polymorphism at the human myelin basic protein gene (MBP). *Human Molecular Genetics*, **1**, 658.

Polymeropoulos MH, Xiao H, Merril CR (1992b) Tetranucleotide repeat polymorphism at the human c-fes/fps proto-oncogene (FES). *Nucleic Acids Research*, **19**, 4018.

Ruano G, Pagliaro EM, Schwartz TR *et al.* (1992) Heat-soaked PCR: an efficient method for DNA amplification with applications to forensic analysis. *BioTechniques*, **13**, 266–274.

Taberlet P, Bouvet J (1994) Mitochondrial DNA polymorphism, phylogeography, and conservation genetics of the brown bear *Ursus arctos* in Europe. *Proceedings of the Royal Society of London* 195–200.

Taberlet P, Griffin S, Goossens B *et al.* (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research*, **16**, 3189–3194.

Taberlet P, Mattock H, Dubois-Paganon C, Bouvet J (1993) Sexing free ranging brown bears *Ursus arctos* using hairs found in the field. *Molecular Ecology*, **2**, 399–403.

Takasaki H, Takenaka O (1991) Paternity testing in chimpanzees with DNA amplification from hairs and buccal cells in wadges. A preliminary note. In: *Primatology Today: Proceedings of the 13th Congress of the International Primatological Society* (eds Ehara A, Kinura T, Takenaka O, Iwamoto M), pp. 613–616. Elsevier, Amsterdam.

Taylor AC (1995) *Molecular Ecology of the Endangered Northern Hairy-nosed Wombat* Lasiorhinus krefftii *and Application to Conservation and Management*. PhD thesis, University of New South Wales, Sydney.

Uchihi R, Tamaki K, Kojima T *et al.* (1992) Deoxyribonucleic Acid (DNA) typing of human leukocyte antigen (HLA) -DQAA1 from single hairs in Japanese. *Journal of Forensic Sciences*, **37**, 853–859.

Walsh PS, Ehrlich HA, Higuchi R (1992) Preferential amplification

of alleles: mechanisms and solutions. *PCR Methods and Applications*, **1**, 241–250.

Weber JL, Kwitek AE, May PE (1990) Dinucleotide repeat polymorphisms at the D16S260, D16S261, D16S265, D16S266, and D16S267 loci. *Nucleic Acids Research* **18**, 4034.

Weber JL, May PE (1990) Dinucleotide repeat polymorphism at the locus D18S35. *Nucleic Acids Research*, **21**, 6465.

Weber JL, May PE (1989) Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *American Journal of Human Genetics*, **44**, 388–396.

Weber JL, May PE, Kwitek AE (1990) Dinucleotide repeat polymorphism at the D16S260, D16S261, D16S265, D16S266, and D16S267 loci. *Nucleic Acids Research*, **13**, 4034.

Woodruff DS (1993) Non-invasive genotyping of primates. *Primates*, **34**, 333–346.

Zhang L, Leeflang EP, Yu J, Arnheim N (1994) Studying human mutations by sperm typing: instability of CAG trinucleotide repeats in the human androgen receptor gene. *Nature Genetics*, **7**, 531–535.

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