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Quantitative polymerase chain reaction analysis of DNA from noninvasive samples for accurate microsatellite genotyping of wild chimpanzees (*Pan troglodytes verus*)

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Abstract

Noninvasive samples are useful for molecular genetic analyses of wild animal populations. However, the low DNA content of such samples makes DNA amplification difficult, and there is the potential for erroneous results when one of two alleles at heterozygous microsatellite loci fails to be amplified. In this study we describe an assay designed to measure the amount of amplifiable nuclear DNA in low DNA concentration extracts from noninvasive samples. We describe the range of DNA amounts obtained from chimpanzee faeces and shed hair samples and formulate a new efficient approach for accurate microsatellite genotyping. Prescreening of extracts for DNA quantity is recommended for sorting of samples for likely success and reliability. Repetition of results remains extensive for analysis of microsatellite amplifications beginning from low starting amounts of DNA, but is reduced for those with higher DNA content.

Keywords: chimpanzees, faeces, microsatellite genotyping, noninvasive samples, paternity, quantitative PCR

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Introduction

Analysis of individual variation at multiple microsatellite loci is a powerful tool for answering questions about genetic diversity, relatedness of individuals and population affiliation (Sunnucks 2000). Noninvasively collected materials such as shed hair, faeces or chewed fruit remnants (wadges) are preferred for genetic analysis of wild primate populations because their collection does not require capture of the animals. The majority of great ape genetic studies using noninvasive samples have relied on the use of shed, or occasionally, plucked hair as the sample material [chimpanzees (Morin *et al*. 1994; Gagneux *et al*. 1997a; Goldberg & Ruvolo 1997; Gonder *et al*. 1997; Constable 2000), gorillas (Garner & Ryder 1996; Field *et al*. 1998) and orang-utans (Muir *et al*. 1994)]. Demonstration of the utility of faeces in genetic studies (Höss *et al*. 1992; Kohn *et al*. 1995; Taberlet *et al*. 1997), along with three recent studies comparing storage and extraction methods of faeces for DNA analysis have made it clear that faecal samples provide a potentially reliable source of genomic DNA for population studies (Wasser *et al*. 1997; Frantzen *et al*. 1998; Flagstad *et al*. 1999). Reports of primate studies utilizing faeces have become more prevalent in recent years (Gerloff *et al*. 1995; Launhardt *et al*. 1998; Constable 2000; Smith *et al*. 2000) although truly comprehensive studies of wild animal populations using noninvasive samples are still few (Taberlet *et al*. 1999).

Several years ago it was noted that special precautions are needed in order to ensure the accuracy of microsatellite genotyping results obtained using low concentration template DNA such as that typically obtained from noninvasive samples (Navidi *et al*. 1992). One type of error encountered is the stochastic amplification of only one of two alleles at a heterozygous locus. If undetected, this 'allelic dropout' leads to the false inference of a homozygous genotype at a locus. A second type of error is that of false alleles resulting from amplification of small amounts of contaminating DNA in competition with similarly small amounts of template DNA. Contamination can be either pervasive, where the sample persistently gives unreliable results, or sporadic, where only occasional irreproducible results are obtained. As a result of the possibility of both types of error, considerable repetition of amplifications and replication of results is necessary to obtain statistical certainty of

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genotypes. Navidi *et al*. (1992) used theoretical considerations to derive the number of repetitions necessary from low concentration templates and concluded that a statistically certain finding of homozygosity should be based upon 10 or more replications, a 'multiple-tubes' approach to genotyping. This recommendation was later refined by a combination of theoretical and limited experimental data, and a guideline of two replications of each allele for heterozygous loci and seven replications for homozygous loci was recommended (Taberlet *et al*. 1996).

In practice, few investigators working with low-concentration DNA from noninvasive samples follow the multiple tubes approach to the recommended extent (Gagneux *et al*. 1997b; Gerloff *et al*. 1999; Kohn *et al*. 1999) because the multiple tubes approach can exhaust finite sample material while also adding significantly to the time and expense of a project. Instead, nonindependent assessments of reliability are often made, such as comparisons of mother–offspring genotypes or comparison of the results from multiple extracts.

Measurement of the DNA content of noninvasive sample extracts can, in principle, allow classification of extracts by DNA content, and thus expedite the genotyping process while increasing the reliability of the results. For example, extracts of high DNA content could be identified and be subject to less replication, extracts containing low amounts of DNA would be subject to rigorous repetition, and extracts containing no detectable DNA would not be used. Unfortunately, conventional fluorimetric assays of DNA content often lack sufficient sensitivity to measure these very dilute samples. In addition, conventional assays cannot distinguish between the DNA of interest and copurified bacterial or plant DNAs that are likely to be present in large amounts in faecal extracts. A more useful measure would be of the amount of amplifiable DNA of interest present in a sample extract.

For this study, the 5′ nuclease assay (Holland *et al*. 1991; Livak *et al*. 1995a) was used for real time quantitative polymerase chain reaction (PCR) amplification (Heid *et al*. 1996; Lie & Petropoulos 1998) of DNA extracts from noninvasive samples. In this assay, a standard PCR is performed, but with the addition into the reagent mix of a 'probe' oligonucleotide that is complementary to a segment of the template DNA to be amplified. The probe is double-labelled with a 'reporter' and a 'quencher' dye at the 5′- and 3′-ends, respectively. While the probe oligonucleotide is intact, the energy from the reporter dye is transferred to the nearby quencher dye, and only the quencher dye fluoresces. During PCR cycles, the *Taq* DNA polymerase enzyme replicates the template DNA and by means of its 5′–3′ exonuclease activity also cleaves the probe it encounters bound to the template DNA. Cleavage of the probe separates the reporter from the quencher dye, and causes an increase in the fluorescence of the reporter dye in solution. Because the probe is present in nonlimiting concentrations, and hybridizes at a higher temperature than the PCR primers, all copies of the template DNA acquire a probe and thus there is a direct correlation between the number of copies of PCR product made and the increase in fluorescence during the exponential increase phase of the PCR. Real-time analysis of the fluorescence levels at each cycle of the PCR allows determination of the fluorescence curve for each sample and for a set of standard templates of known DNA amounts. The cycle at which fluorescence reaches an arbitrary threshold level (the C_t) in the exponential phase is determined for each standard and unknown sample, and the C_t values of the standards are plotted against the amount of DNA to create a standard curve. Comparison of the sample C_t values with C_t values from a standard curve allows accurate determination of the starting DNA template amount over at least 5 orders of magnitude (Lie & Petropoulos 1998).

As part of a long-term study examining reproductive strategies of male and female chimpanzees (*Pan troglodytes verus*) in West Africa, we are in the process of assembling multilocus microsatellite genotypes from three communities of habituated chimpanzees living in the Taï National Park, Côte d'Ivoire. For this project we rely on the use of DNA from faeces and shed hair. In this study we addressed four questions. (i) How much amplifiable nuclear DNA is present in an extract from a sample of faeces or shed hair? (ii) How difficult is it to amplify microsatellite loci from extracts of varied DNA content, and are there different success rates between loci? (iii) How is the rate of allelic dropout related to the starting amount of DNA in an amplification? And finally, (iv) can quantification of DNA increase the speed and accuracy of microsatellite genotyping when using noninvasive samples?

Materials and methods

Sampling

The Taï chimpanzees have been the subject of field observation for over 20 years (Boesch & Boesch-Achermann 2000). Faecal samples were collected from individually known chimpanzees shortly after defecation. Approximately 5 g of faecal sample was placed in a 50-mL screw-top tube containing 20 g of silica gel beads (Sigma) (Wasser *et al*. 1997) and stored at ambient temperature for several weeks. Samples were transferred to fresh tubes of silica upon receipt in the laboratory and kept at 4 °C. Whenever possible, multiple samples from different days were collected for each individual. Faecal samples were stored for 2–18 months prior to extraction. Shed hair was collected from recently vacated night nests, placed individually in glassine envelopes and stored in an airtight container with desiccant. Upon receipt in the laboratory the desiccant was replaced and the

hair samples were stored at −80 °C. Hairs used in this study were stored for an average of 8 years.

DNA extractions

Genomic DNA was extracted from 100 mg of dried faeces using the QIAamp® DNA Stool Kit (Qiagen) according to manufacturer's instructions with the following modifications. Dried faecal samples were rehydrated prior to extraction by placing samples for 72 h in a closed glass vessel at 4 °C with 95% humidity. The rehydrated samples were incubated at room temperature for 30–60 min in ASL buffer, and the final elution of DNA into buffer AE occurred for 20–30 min. Ten samples and two negative controls were processed at one time. DNA was recovered in 200 µL of elution buffer, aliquoted and stored at −20 °C. Genomic DNA was extracted from an ≈ 3-mm segment of the root end of individual shed hairs using a simple process of proteinase K digestion in a PCR-compatible buffer (Allen *et al*. 1998; Vigilant 1999), also resulting in a final volume of 200 µL stored in the same manner as above.

Microsatellite amplification and analysis

Primer sequences, both original and modified, for the nine microsatellite loci have been described elsewhere (Bradley *et al*. 2001). Individual PCR amplifications were performed in 15 µL reactions containing either 2 µL of DNA extract from faeces or 5 µL of DNA extract from hair, 1× PCR buffer (10 mM Tris–HCl, pH 8.3; 50 mM KCl), 2 mM $MgCl_2$, 6 µg bovine serum albumin (BSA), 250 µM each dNTP, 200 nM each primer and 0.375 U Amplitaq Gold DNA polymerase (Perkin–Elmer). Amplification conditions on a PTC-200 thermocycler (MJ Research) were: initial denaturation at 95 °C for 3 min; 45 (faeces DNA) or 50 (hair DNA) cycles of 30 s at 95 °C, 30 s at 55 or 60 °C, 30 s at 72 °C, and a final extension of 30 min at 72 °C. The 5′-end of the forward primer was fluorescently labelled, and amplification products were separated using capillary electrophoresis (ABI PRISM 310). Alleles were sized relative to an internal size standard (HD400 with ROX label) using GENESCAN 2.0 (Perkin-Elmer Applied Biosystems).

Quantitative PCR

The 5′ nuclease assay targeted an 81-bp portion of the c-*myc* proto-oncogene from mouse (Accession no. X01023) and human (Accession no. J00120) and design followed published guidelines (Livak *et al*. 1995b; Morin *et al*. 1999). The chimpanzee c-*myc* gene has also been sequenced (Accession no. M38057), and the sequences of the primers and probes for this assay are perfectly homologous to the chimpanzee sequence. Briefly, the primers were designed to anneal at a predicted 59 °C and to closely flank an oligonucleotide probe annealing at a predicted 10 °C above the annealing temperature (T_a) of the primers. The primers and probes were selected using the software program PRIMER EXPRESS™ (v. 1.0; PE Biosystems, Foster City, CA, USA). Probes were synthesized with a 5′-VIC reporter dye and a 3′-TAMRA quencher dye (PE Biosystems, Germany, and Metabion GMBH, Germany). The oligonucleotide sequences were: forward primer (CMYC_E3_F1U1) GCCAGAGGAGGAACGAGCT, reverse primer (CMYC_E3_R1U1) GGGCCTTTTCATTGTTTTCCA and probe (CMYC_E3_TMV) VIC-TGCCCTGCGTGACCAGATCC-TAMRA. The 5′ nuclease assay was performed in 20 µL PCR containing 1× 'TaqMan Universal PCR Mastermix' (PE Biosystems), 300 nM each primer, 200 nM probe, 8 µg BSA (Boehringer-Mannheim) and 2 µL DNA extract. PCR amplification was performed in an ABI Prism™ 7700 Sequence Detector (PE Biosystems), with initial incubations for 2 min at 50 °C and 10 min at 95 °C, followed by 50 cycles of 95 °C for 15 s and 59 °C for 30 s. Analysis was performed using the ABI Prism™ 7700 Sequence Detector software, and quantities were checked independently using standard curves and calculations in Microsoft EXCEL spreadsheets.

Triplicate sets of standards of known DNA amount were included with each set of samples amplified with a single preparation of the PCR reagent mix, and, if necessary, the occasional significantly outlying points (e.g. a failed PCR) in the resulting standard curve were deleted so they would not change the slope of the line. A standard curve was rejected if the correlation coefficient of the trendline was < 0.95. The standard curve DNAs consisted of 11 dilutions of ultrapure human placental DNA (Sigma D-4642) quantified first by absorbance (A_{260}) in a spectrophotometer, and then by using the 5′ exonuclease assay described here, using the PE Biosystems standard curve (Part no. 401970) as a reference. DNA amounts in the standard were (in 5 µL): 40 ng, 10 ng, 2.5 ng, 1.25 ng, 625 pg, 312 pg, 156 pg, 78 pg, 39 pg, 19 pg, 10 pg. A 'no-template control' (NTC) was included with each standard curve, and 3–6 additional NTCs were included with each plate of samples. DNA amounts for each assay were calculated from the slope and Y-intercept (Y_{int}) of the trendline from the standard curve, plotted as the log of the DNA amounts vs. the C_t values: DNA amount = $10^{((C_t - Y_{int})/\text{slope})}$.

Results

DNA quantification

The average C_t values from nine replicate standard curves from three experiments using the c-*myc* nuclear DNA assay are shown in Fig. 1. All of the data points from these curves were used to calculate the average values, producing a curve with a trendline correlation coefficient of 0.9979. In

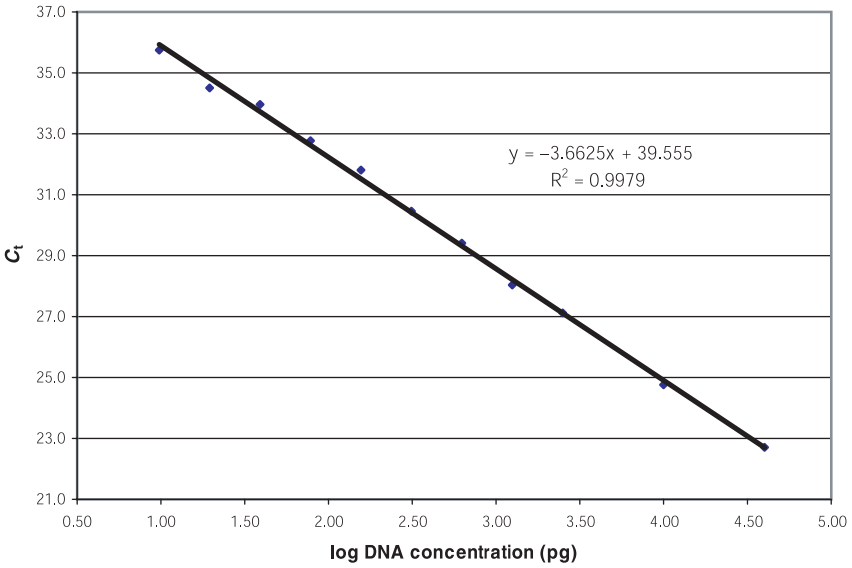

Fig. 1 Plot of the average C_t values for each DNA concentration from nine replicate standard curves, from three independent PCR experiments using the c-*myc* 5′ nuclease assay.

DNA amount		C_t values		Calculated DNA amount	
pg	Log (pg)	Average	SD	Average (pg)	SD
40 000	4.602	22.71	±0.192	40 098	±5061
10 000	4.000	24.76	0.537	11 539	4172
2 500	3.398	27.12	0.580	2 641	994
1 250	3.097	28.04	0.525	1 466	480
625	2.796	29.41	0.538	619	203
313	2.495	30.46	0.376	312	73
156	2.194	31.81	0.549	137	42
78	1.893	32.77	0.810	79	35
39	1.592	33.96	0.901	39	23
20	1.291	34.50	0.560	25	8
10	0.990	35.75	0.999	12	5

Table 1 Observed C_t values and standard deviations from nine replicate standard curves summarized in Fig. 1. Calculated DNA concentrations are based on the trendline equation in Fig. 1

general, trendlines for individual experiments (triplicate curves) were in excess of 0.98, either with all points included, or occasionally one or two points removed if a particular DNA concentration in the standard curve had points significantly away from the trendline produced by the rest of the data (usually representing a failed PCR). The standard deviations of the individual C_t values for each DNA concentration for the nine standard curves in Fig. 1 are shown in Table 1, along with the means and standard deviations for the calculated DNA concentrations from the average standard curve trendline equation.

The c-*myc* 5′ nuclease assay was used to quantify the amount of amplifiable single-copy DNA present in 107 faecal and 290 single shed hair DNA extracts. Results are listed in Appendix I (this appendix is available from the Molecular Ecology website, URL http://www.blackwell-science.com/products/journals/suppmat/mec/mec1308/mec1308sm.htm). The average DNA concentration found in faecal extracts was 192 pg/μL (range: 0–2550), and so an entire 200-μL extract contained an average of 38.4 ng and up to 510 ng of DNA. Hair extracts contained an average of 4.4 pg/μL (range: 0–228) DNA, and so the average and maximum amount of DNA recovered in 200 μL from a single shed hair were 0.88 and 45.6 ng, respectively. The distribution of template DNA concentrations is shown in Fig. 2. The majority (79%) of the extracts from shed hair contain no amplifiable DNA, whereas only 7% of the faecal extracts measured as zero. Fewer than 2% of the hair extracts contain > 50 pg/μL, while in contrast nearly half (49%) of the extracts from faeces contain > 50 pg/μL.

For comparison, 21 freshly plucked human hairs were also extracted, and were found to contain an average of 326 pg/μL DNA (range: 24–1202), which is > 15 times the average amount of DNA (21 pg/μL) found in the 61 chimpanzee hairs with nonzero results. Because the approximate amount of nuclear DNA present in a single

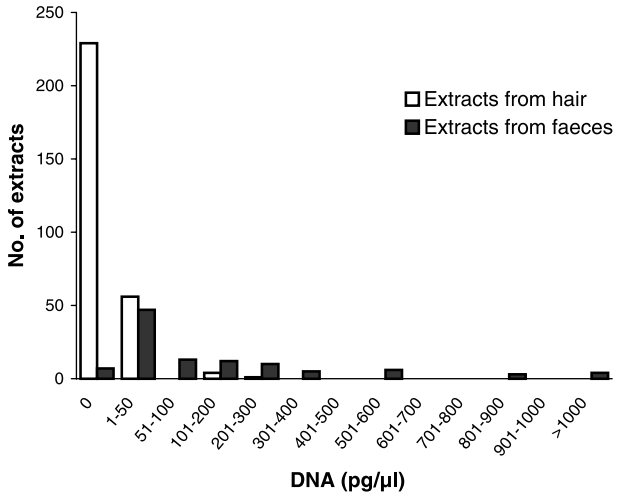

Fig. 2 DNA concentration measured in 107 extracts from faeces and 290 extracts from single hairs. All extracts contained a total volume of 200 µL.

diploid cell is ≈ 7 pg (Navidi *et al*. 1992), this implies that for the 21% of the shed chimpanzee hair extracts that contain DNA, the total amount contained is on average equivalent to 600 diploid cells. The average faecal extract contained 38 400 pg, equivalent to almost 5500 cells. Because each faecal extraction began with ≈ 0.1 g of faeces, it can be inferred that, on average, ≈ 55 000 cells were contained in 1 g of dried chimpanzee faeces.

Microsatellite amplification from faecal extracts

The success rate and accuracy of microsatellite amplification at nine loci using 90 of the 107 faecal extracts was examined. Incomplete genotypes were produced using the other 17 faecal extracts and hence the results are not used in this analysis. All genotypes used in this analysis were the result of using multiple DNA extracts for each individual and were scored using the multiple tubes criteria for replication of results (Taberlet *et al*. 1996). The individuals studied include 15 mother–offspring pairs, and no evidence of non-Mendelian inheritance (nonsharing of alleles) was seen at any locus. The dependence of PCR success on the starting template concentration illustrated in Fig. 3 shows that a substantial number of positive PCR results may be obtained from even a small starting amount of template.

The frequency of allelic dropout, the amplification of only one of two alleles at a heterozygous microsatellite locus, was calculated and compared with starting amount of DNA. Loci were classified as heterozygous for an individual if each allele was observed at least twice from a minimum of two separate amplifications. Allelic dropout is observed most frequently in amplifications that begin with a low starting amount of template (Fig. 3). Amplifications beginning from < 100 pg of template produce a PCR product in two-thirds of attempts, but on average almost half (49%) of these products exhibited allelic dropout.

The extent of amplification failure and dropout at individual microsatellite loci is described in Table 2. Primers were redesigned for four of the loci in order to obtain products of shorter length; in these cases data from both primer sets are presented (Bradley *et al*. 2001). The success rate of amplification at individual loci ranged from 65 to 97%, with an average of 79% calculated from > 1800 individual PCRs. The extent of dropout at heterozygous loci averaged 24%, as calculated from > 1300 reactions. The extent of dropout at the sole trinucleotide locus, *D9s910*, at 19% was

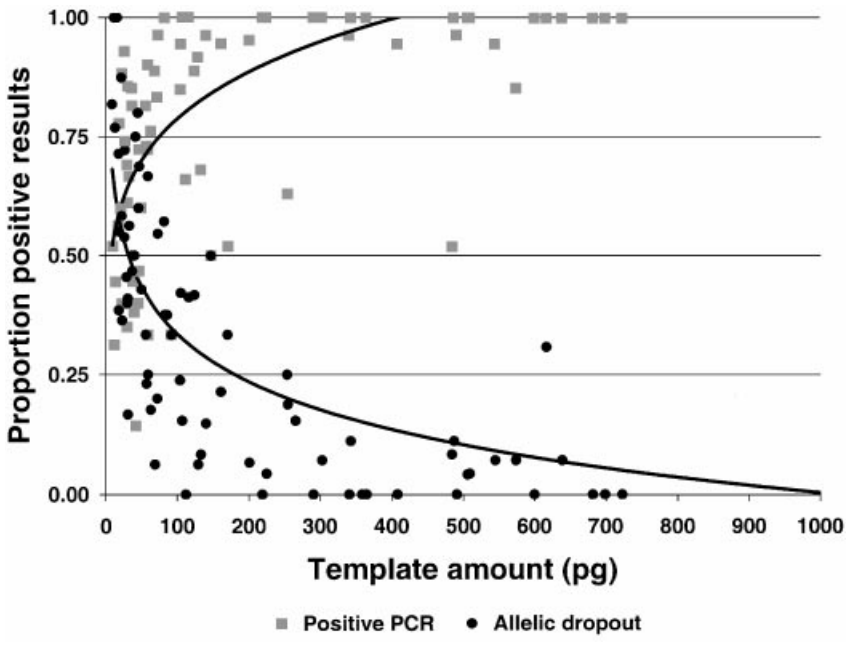

Fig. 3 Relationship between the initial amount of template DNA in the PCR and both the proportion of PCRs with amplification product (grey squares) and the proportion of PCRs with allelic dropout (black circles). Each point represents results from one faecal DNA extract. PCR amplifications of microsatellite loci were attempted from each of the 90 extracts an average of 21 times. For each extract an average of 15 amplifications of heterozygous loci was examined for allelic dropout. The relationship between template amount and positive PCR results is described by the curve with the equation $y = 0.3595x^{0.1702}$. The allelic dropout data produces a curve with the equation $y = -0.1439 \ln(x) + 0.9977$.

Table 2 Proportion of positive polymerase chain reaction and dropout per locus

Locus	Primer pair	Product size range	Positive PCR	Dropout
D2s1326	F-R	222–266	75% (95/126)	15% (11/72)
	F-R2	174–218	74% (73/99)	31% (21/67)
D7s817	F-R	164–200	84% (104/124)	35% (33/95)
	F2-R	120–156	88% (87/99)	12% (10/81)
D11s2002	F-R	238–266	80% (78/98)	34% (21/61)
	F-R2	144–172	97% (95/98)	26% (31/118)
D2s1329	F-R	171–223	71% (111/156)	27% (23/84)
	F2-R2	154–206	80% (61/76)	19% (12/63)
D7s2204	F-R	229–265	65% (133/205)	32% (41/128)
vwf	F-R	116–148	85% (187/221)	20% (17/87)
D9s910	F-R	101–122	92% (153/166)	19% (31/160)
D12s66	F-R	146–178	87% (182/209)	23% (28/121)
D5s1470	F-R	170–190	92% (128/139)	21% (40/191)
Total			79% (1487/1886)	24% (319/1328)

Table 3 Criteria for genotype results with high confidence (99% certainty) for various categories of initial DNA template amount

Template (pg/rxn)	Dropout/total types	Repetition needed
< 25	81/119 = 68%	Do not use
26–100	156/369 = 42%	7
101–200	48/184 = 26%	4
201 +	34/656 = 5.2%	2

within the range (12–35%) exhibited by the tetranucleotide loci. A total of 12 cases of anomalous, nonreproducible results was observed in 1328 reactions, leading to an estimated error rate below 1%.

Criteria for genotype accuracy based upon template concentration

The relationship between template concentration and allelic dropout shown in Fig. 3 makes it possible to devise empirically based criteria for accurate genotyping. In Table 3 the average amount of allelic dropout observed at heterozygous loci for various categories of starting template amount is listed. The dropout rate varies dramatically between categories, and is quite high for templates below 50 pg. Following the example of Taberlet *et al*. (1996), the experimentally derived dropout rate is used to calculate the amount of repetition necessary for correct results at the 99% certainty level. For example, seven replications, calculated as $(0.42)^7$, are necessary to reach this certainty level for the category of 26–100 pg of template. Four replications are needed to score with high confidence homozygous loci amplified using template concentrations of 101–200 pg per reaction, whereas two are needed for template amounts > 201 pg. For the category of lowest template amount (< 25 pg), the 68% dropout leads to an estimate of a minimum of 12 necessary replications. However, the curve describing the extent of dropout in relation to template amount in Fig. 3 is extremely steep at low (< 25 pg) amounts of template, suggesting that some templates can exhibit dropout to such a high extent that prudence argues against their use. It is also possible to calculate the predicted dropout rate given any initial template amount using the equation for the line describing dropout in Fig. 3. Because this line is an approximate fit to all of the data, we preferred to calculate average rates of dropout using only data points within that category, as described above.

The recommendations summarized in Table 3 contrast greatly with the recommendations currently in place for genotyping using noninvasive sample material, which consist of an initial scoring of four PCRs for all loci, followed by an additional three or more replications of results from apparently homozygous loci, with additional reactions as required to resolve any ambiguities. This procedure has the disadvantage of requiring a large total number of reactions, and hence substantial expenditure of time, money and sample material. In addition, unless an extract is quickly recognized as producing few PCR products, many reactions may be attempted from extracts of poor quality. A flow-chart illustrating a refined approach for efficient and accurate microsatellite genotyping from noninvasive samples is given in Fig. 4. Characterization of the DNA extracts by DNA quantification, in this figure termed 'extract sorting', is the crucial step for gauging the likely success and accuracy of amplifications from these extracts, rather than a trial and error approach involving many PCRs.

Discussion

We designed a 5′ nuclease assay for nuclear DNA that accurately measures the amount of amplifiable single-copy DNA obtained from chimpanzee hair and faecal samples. In addition, the primers and probe were designed based on

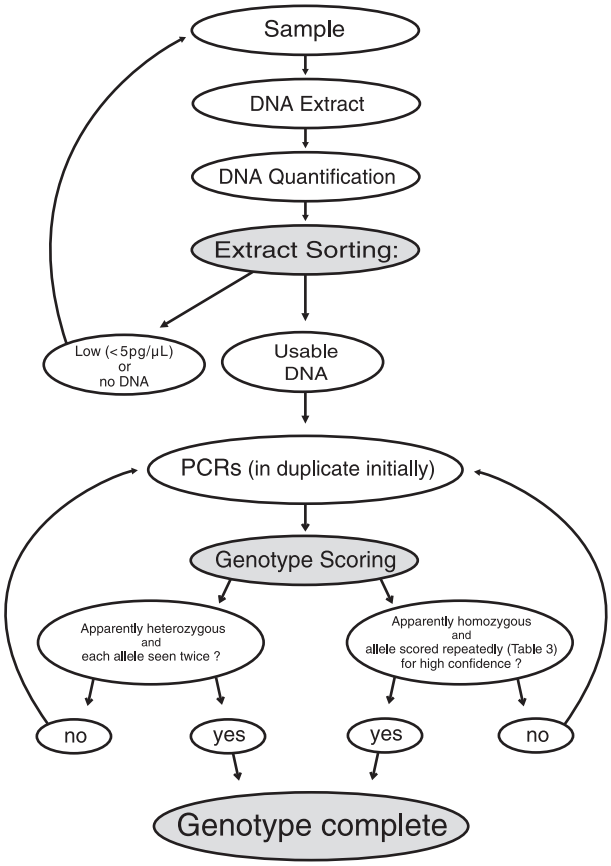

Fig. 4 Flowchart illustrating the recommended procedure for efficient and accurate microsatellite genotyping using DNA from noninvasive, as well as other type samples.

homologous sequences of mouse and human, with only one nucleotide mismatch between the mouse and human sequence in each of the primers (data not shown), therefore this assay should be useful for quantifying DNA from diverse vertebrates, and from diverse sample types, such as minute and degraded ancient and noninvasive samples. The assay is simple and completed in a single tube, so that risk of contamination is minimized when proper DNA handling procedures are followed (Kwok 1990). Although the equipment needed for real-time analysis of PCR is not yet standard in most molecular ecology laboratories, the assay reagents are very stable, and can be easily assembled and transported to molecular medicine laboratories where the equipment is now fairly standard for gene expression studies, and no modification of the instrumentation is typically necessary.

Potential sources of error affecting this assay include variation in PCRs and stochastic processes associated with a small starting copy number (Lockey *et al*. 1998; Raeymaekers 1999), but our analysis of a set of 11 standard DNA concentrations analysed nine times from three separate experiments shows that the mean values are very close to the expected values, and correlation coefficients for the standard curves are high (typically > 0.98), therefore the amount of tube-to-tube variation among replicates is minimal.

Other fluorimetric methods (e.g. Gagneux *et al*. 1997b) approach the sensitivity of the 5′ exonuclease assay for measuring double-stranded DNA, but lack the specificity for a particular target sequence, and therefore measure total DNA content. This can be misleading when the DNA content includes contaminating DNA (e.g. plant and bacterial DNA in faecal samples), and also when samples contain PCR inhibitors.

Although the presence of BSA in the 5′ nuclease assay is not standard practice, we introduced this agent because it is typically used in our laboratory in nonquantitative PCR applications, such as the amplification of microsatellite loci, and so its presence in the 5′ nuclease assay ensures comparable results in cases in which inhibitory compounds are present in the DNA extraction. BSA is presumed to function by binding inhibitors of the PCR (Pääbo 1990). Because inhibitors reduce the efficiency of PCR, they produce a situation similar to that of low template concentration, and so, despite possibly containing adequate concentrations of DNA, these samples would be measured by this functional assay as low-concentration samples and would in addition mimic low-concentration DNA samples in amplifying microsatellite loci with reduced success and accuracy. DNA extracted from faeces has been reported to contain inhibitors of PCR (Monteiro *et al*. 1997), and we inferred the presence of inhibitors in several faecal DNA extracts that failed to amplify without the inclusion of BSA in the reaction (data not shown).

Application of the 5′ nuclease assay allowed us to quantify the amount of DNA present in extracts from chimpanzee hair and faeces. Faeces is clearly a much better source of DNA than hair, with an extract from faeces containing an average total of 38.4 ng of DNA, whereas the minority of single hairs that contained detectable amounts of DNA had an average total content of only 4.2 ng. The use of the assay to identify the hairs with no amplifiable DNA eliminated fruitless attempts to genotype these hairs. Results from hairs collected more recently and stored for as short a time as 10 months were similar (data not shown), leading us to conclude that it is more likely that most shed chimpanzee hairs contained very little or no DNA upon collection rather than that the DNA has degraded upon long-term storage in the freezer. In contrast to nuclear DNA, mitochondrial DNA (mtDNA) is present in many copies per cell and has therefore been the target of many investigations using noninvasive samples. A 5′ exonuclease assay targeting a portion of the mitochondrial 12*S* ribosomal RNA (rRNA) gene was able to detect still lower concentrations of total genomic DNA than the c-*myc* assay described here, and in fact several samples had positive readings despite containing no detectable DNA using

the c-*myc* assay (data not shown). The excess of mtDNA relative to nuclear DNA is most likely to be the result of different initial amounts of these DNAs in the samples, and more speculatively, perhaps also the consequence of differential stability of the two types of DNA during storage. Quantification of DNA for mtDNA studies is also essential to avoid errors arising from nucleotide misincorporation in the first cycles of amplification from few starting molecules (Handt *et al*. 1996). Thus, a strategy such as the one outlined here, of quantification followed by appropriate repetition, should also be useful for mtDNA studies using noninvasive samples.

A strong dependence of PCR success on the initial template amount was demonstrated, but with a surprising number of positive reactions (59%) produced from reactions containing very low (< 25 pg) amounts of template DNA. Furthermore, we examined in detail, using results from > 1300 positive amplifications, the incidence of allelic dropout, the error most commonly encountered when amplifying microsatellite loci using DNA extracted from noninvasive samples. The information from DNA quantification was used to develop refined criteria for accurate microsatellite genotyping. The crucial step is the classification of extracts by DNA content, allowing decisions to be made about which extracts to continue using and the amount of repetition necessary for statistically reliable results. It is possible to adjust the amount of extract used in an amplification reaction to achieve a higher template concentration, but this poses the risk of exhausting the sample material before genotyping is complete. In addition, increasing the amount of extract used may not necessarily improve success as it may also simultaneously increase the concentration of PCR inhibitors. Our standard protocol used 2 μL of faecal extract per amplification, so that from a total volume of 200 μL up to 100 reactions could be performed. In contrast, for hair extracts 5 μL of extract per reaction was used, permitting a total of only 40 reactions, which would likely be insufficient to complete the genotyping of 10 loci using the unmodified multiple tubes approach.

Application of the new genotyping procedure described in Fig. 4 has increased the efficiency of the chimpanzee genotyping project in our laboratory. Our procedure has changed from an initial extraction in duplicate of each faecal sample, followed by triplicate PCRs at each locus, to one in which single extracts are prepared from each hair or faeces sample and quantified. Only the extracts with nonzero DNA concentrations, representing only 31% of the hair extracts but 93% of the faecal extracts, are used to produce duplicate PCRs at each locus. Extracts with particularly low DNA concentrations (< 5 pg/μL) are put aside and a new extract is prepared and quantified for use in its place. It is difficult to calculate exactly the reduction in repetition possible using the new regimen, but for our project the reduction from double to single extractions, a particularly time-consuming procedure, and the switch from initial triplicate to duplicate PCRs has resulted in a threefold reduction in the number of PCRs attempted.

It is worth noting that an earlier study by Taberlet *et al*. (1996) found that ≈ 56 pg of template DNA represented a critical threshold value below which allelic dropout could reach high proportions. The consistency between that value and those found in this study, in which amounts below 25 pg were found to be so unreliable as to be unusable and 25–100 pg is a category in which seven repetitions of results are needed, lends support to the idea that the categories of DNA amounts presented here should be applicable to other microsatellite genotyping studies. A similar relationship between template amount and extent of dropout has also been seen in an ongoing microsatellite genotyping project of mountain gorilla faeces in our laboratory (B. Bradley, personal communication).

The presence of errors in microsatellite genotypes can have disastrous consequences for studies attempting identification of individuals or paternity assignment, because these studies draw conclusions based upon the presence of mismatches between individuals. The rate of errors of unknown cause was estimated at < 1% in this study, and the use of tri- and tetranucleotide repeat loci eliminated to a large extent the incidence of stutter bands that complicate analysis of dinucleotide repeat loci. In conclusion, application of the 5′ nuclease assay presented here, in conjunction with the 'modified multiple tubes' procedure, can aid in the efficient control of allelic dropout, the main source of error encountered in microsatellite genotyping studies, allowing accurate results to be obtained with less time, effort and expenditure of materials.

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References

Allen M, Engström AS, Meyers S *et al*. (1998) Mitochondrial DNA sequencing of shed hairs and saliva on robbery caps: sensitivity and matching probabilities. *Journal of Forensic Science*, **43**, 453–464.

Boesch C, Boesch-Achermann H (2000) *The Chimpanzees of the Taï Forest*. Oxford University Press, Oxford.

Bradley BJ, Boesch C, Vigilant L (2001) Identification and redesign of human microsatellite markers for genotyping wild chimpanzee (*Pan troglodytes verus*) and gorilla (*Gorilla gorilla gorilla*) DNA. from faeces. *Conservation Genetics*, **1**, 289–292.

Constable J (2000) *Paternity analysis and reproductive success in the Gombe chimpanzees*. PhD Thesis, University of Minnesota, USA.

Field D, Chemnick L, Robbins M, Garner K, Ryder O (1998) Paternity determination in captive lowland gorillas and orangutans and wild mountain gorillas by microsatellite analysis. *Primates*, **39**, 199–209.

Flagstad O, Roed K, Stacy JE, Jakobsen KS (1999) Reliable noninvasive genotyping based on excremental PCR of nuclear DNA purified with a magnetic bead protocol. *Molecular Ecology*, **8**, 879–883.

Frantzen MA, Silk JB, Ferguson JW, Wayne RK, Kohn MH (1998) Empirical evaluation of preservation methods for faecal DNA. *Molecular Ecology*, **7**, 1423–1428.

Gagneux P, Boesch C, Woodruff DS (1997b) Microsatellite scoring errors associated with noninvasive genotyping based on nuclear DNA amplified from shed hair. *Molecular Ecology*, **6**, 861–868.

Gagneux P, Woodruff DS, Boesch C (1997a) Furtive mating in female chimpanzees. *Nature*, **387**, 358–359.

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

Gerloff U, Hartung B, Fruth B, Hohmann G, Tautz D (1999) Intracommunity relationships, dispersal pattern and paternity success in a wild living community of Bonobos (*Pan paniscus*) determined from DNA analysis of faecal samples. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, **266**, 1189–1195.

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

Goldberg TL, Ruvolo M (1997) Molecular phylogenetics and historical biogeography of east African chimpanzees. *Biological Journal of the Linnean Society*, **61**, 301–324.

Gonder MK, Oates JF, Disotell TR *et al.* (1997) A new west African chimpanzee subspecies? *Nature*, **388**, 337.

Handt O, Krings M, Ward RH, Paabo S (1996) The retrieval of ancient human DNA sequences. *American Journal of Human Genetics*, **59**, 368–376.

Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. *Genomic Research*, **6**, 986–994.

Holland PM, Abramson RD, Watson R, Gelfand DH (1991) Detection of specific polymerase chain reaction product by utilizing the 5′ to 3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proceedings of the National Academy of Sciences of the USA*, **88**, 7276–7280.

Höss M, Kohn M, Pääbo S, Knauer F, Schröder W (1992) Excrement analysis by PCR. *Nature*, **359**, 199.

Kohn M, Knauer F, Stoffella A, Schröder W, Pääbo S (1995) Conservation genetics of the European brown bear — a study using excremental PCR of nuclear and mitochondrial sequences. *Molecular Ecology*, **4**, 95–103.

Kohn MH, York EC, Kamradt DA *et al.* (1999) Estimating population size by genotyping faeces. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, **266**, 657–663.

Kwok S (1990) Procedures to minimize PCR-product carry-over. In: *PCR Protocols* (eds Innis MA, Gelfand DH, Sninsky JJ, White TJ), pp. 142–158. Academic Press, San Diego, CA.

Launhardt K, Epplen C, Epplen JT, Winkler P (1998) Amplification of microsatellites adapted from human systems in faecal DNA of wild Hanuman langurs (*Presbytis entellus*). *Electrophoresis*, **19**, 1356–1361.

Lie YS, Petropoulos CJ (1998) Advances in quantitative PCR technology: 5′ nuclease assays. *Current Opinions in Biotechnology*, **9**, 43–48.

Livak K, Flood SJA, Marmaro J, Giusti W, Deetz K (1995a) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods and Applications*, **4**, 357–362.

Livak K, Marmaro J, Flood S (1995b) *Guidelines for Designing Taqman™ Fluorogenic Probes for 5′ Nuclease Assay*. PE Applied Biosystems, Foster City, CA.

Lockey C, Otto E, Long Z (1998) Real-time fluorescence detection of a single DNA molecule. *Biotechniques*, **24**, 744–746.

Monteiro L, Bonnemaison D, Vekris A *et al.* (1997) Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. *Journal of Clinical Microbiology*, **35**, 995–998.

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

Morin PA, Saiz RS, Monjazeb A (1999) High-throughput single nucleotide polymorphism genotyping by fluorescent 5′ exonuclease assay. *Biotechniques*, **27**, 538–552.

Muir C, Galdikas BMF, Beckenback AT (1994) Genetic variability in orangutans. In: *The Neglected Ape* (ed. Nadler REA), pp. 267–272. Plenum Press, New York.

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.

Pääbo S (1990) Amplifying ancient DNA. In: *PCR Protocols* (eds Innis MA, Gelfand DH, Sninsky JJ, White TJ), pp. 159–166. Academic Press, San Diego, CA.

Raeymaekers L (1999) General principles of quantitative PCR. In: *Methods in Molecular Medicine* (eds Kochanowski B, Reischl U), pp. 31–41. Humana Press Inc., Totowa, NJ.

Smith KL, Alberts SC, Bayes MK *et al.* (2000) Cross-species amplification, non-invasive genotyping, and non-Mendelian inheritance of human STRPs in Savannah baboons. *American Journal of Primatology*, **51**, 219–227.

Sunnucks P (2000) Efficient genetic markers for population biology. *Trends in Ecology and Evolution*, **15**, 199–203.

Taberlet P, Camarra J-J, Griffin S *et al.* (1997) Noninvasive genetic tracking of the endangered Pyrenean brown bear population. *Molecular Ecology*, **6**, 869–876.

Taberlet P, Griffin S, Goossens B *et al.* (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research*, **24**, 3189–3194.

Taberlet P, Waits LP, Luikart G (1999) Noninvasive genetic sampling: look before you leap. *Trends in Ecology and Evolution*, **14**, 323–327.

Vigilant L (1999) An evaluation of techniques for the extraction and amplification of DNA from naturally shed hairs. *Biological Chemistry*, **380**, 1329–1331.

Wasser SK, Houston CS, Koehler GM, Cadd GG, Fain SR (1997) Techniques for application of faecal DNA methods to field studies of Ursids. *Molecular Ecoogy*, **6**, 1091–1097.

The authors are interested in the use of molecular genetic techniques for the study of wild primate populations sampled in a noninvasive manner, and the integration of genetic information with data obtained from observational studies. P. Morin is a visiting research scientist in the Department of Evolutionary Genetics, and founder of a new Conservation Genetics Laboratory. K. Chambers is a postdoctoral fellow and C. Boesch is the director of the Department of Primatology and the Taï Chimpanzee Project. L. Vigilant is a research scientist directing the molecular genetics projects in the Department of Primatology.

Supplementary material

The following material is available from http://www.blackwell-science.com/products/journals/suppmat/mec/mec1308/mec1308sm.htm

Appendix I

Amount of DNA in each extract measured by the 5′ nuclease assay. Faecal extracts with names in bold were not used in the analysis of PCR success and allelic dropout rates.