Molecular Ecology Resources (2009) **9**, 28–36 doi: 10.1111/j.1755-0998.2008.02387.x

TECHNICAL ADVANCES

Two-step multiplex polymerase chain reaction improves the speed and accuracy of genotyping using DNA from noninvasive and museum samples

M. ARANDJELOVIC, K. GUSCHANSKI, G. SCHUBERT, T. R. HARRIS, O. THALMANN, H. SIEDEL and L. VIGILANT

Max Planck Institute for Evolutionary Anthropology, Deutscher Platz 6, D-04103 Leipzig, Germany

Abstract

Many studies in molecular ecology rely upon the genotyping of large numbers of low-quantity DNA extracts derived from noninvasive or museum specimens. To overcome low amplification success rates and avoid genotyping errors such as allelic dropout and false alleles, multiple polymerase chain reaction (PCR) replicates for each sample are typically used. Recently, two-step multiplex procedures have been introduced which drastically increase the success rate and efficiency of genotyping. However, controversy still exists concerning the amount of replication needed for suitable control of error. Here we describe the use of a two-step multiplex PCR procedure that allows rapid genotyping using at least 19 different microsatellite loci. We applied this approach to quantified amounts of noninvasive DNAs from western chimpanzee, western gorilla, mountain gorilla and black and white colobus faecal samples, as well as to DNA from ~100-year-old gorilla teeth from museums. Analysis of over 45 000 PCRs revealed average success rates of > 90% using faecal DNAs and 74% using museum specimen DNAs. Average allelic dropout rates were substantially reduced compared to those obtained using conventional singleplex PCR protocols, and reliable genotyping using low (< 25 pg) amounts of template DNA was possible. However, four to five replicates of apparently homozygous results are needed to avoid allelic dropout when using the lowest concentration DNAs (< 50 pg/reaction), suggesting that use of protocols allowing routine acceptance of homozygous genotypes after as few as three replicates may lead to unanticipated errors when applied to low-concentration DNAs.

Keywords: black and white colobus, chimpanzee, genotyping errors, gorilla, low-quantity DNA, multiplex pre-amplification method

Received 2 April 2008; revision accepted 29 May 2008

Introduction

Methodological advances in the last decade have made low-quality DNA samples such as faeces increasingly viable for use in genetic studies of wild and even extinct animal populations. Improved collection and storage methods have resulted in an increase in the amount of DNA preserved in samples (Frantzen *et al*. 1998; Bayes *et al*. 2000; Murphy *et al*. 2002; Roon *et al*. 2003; Nsubuga *et al*. 2004; Roeder *et al*. 2004). DNA extraction methods have also been refined to improve yield and decrease inhibitor concentration (Flagstad *et al*. 1999; Wehausen *et al*. 2004; Puechmaille *et al*. 2007). The DNAs are then usually subject to microsatellite genotyping to determine individual identities, ascertain parentage, or assess diversity levels (for example, Kohn *et al*. 1999; Vigilant *et al*. 2001). Due to the use of low-copy number DNAs as templates for the polymerase chain reactions (PCR, Navidi *et al*. 1992), a multiple-tube approach has been widely adopted by researchers to guard against allelic dropout (misidentification of loci as homozygous due to the chance amplification of only one of two heterozygous alleles) and

Correspondence: Mimi Arandjelovic, Fax: +49-341-3550-119; E-mail: arandjel@eva.mpg.de
M. Arandjelovic and K. Guschanski contributed equally to this work.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

false alleles (mistyping due to random contamination, PCR slippage artefacts or other sources) (Taberlet *et al*. 1996; Taberlet *et al*. 1999; Broquet & Petit 2004).

The multiple-tube approach attempts to ensure that the correct genotype is obtained by requiring as many as seven independent PCRs from a given extract for acceptance of a homozygous genotype (Taberlet *et al*. 1996). In general, the lower the amount of template in the PCR, the more prone it is to genotyping errors and the more PCR replicates are required. In order to work efficiently, researchers have attempted to minimize the number of replicates necessary by quantifying the amount of DNA in extracts (Morin *et al*. 2001; Ball *et al*. 2007) or by calculating a quality index for each sample (Miquel *et al*. 2006). More recently, it has been suggested that as few as three independent PCRs may suffice for accurate genotyping results (Hansen *et al*. 2008).

The use of multiple PCR replicates can result in the sample becoming exhausted if many loci are analysed, and has additional costs in money, effort and time. To more effectively use template DNAs, multiplex PCR procedures, in which several loci are co-amplified in a single reaction, have been devised (Chamberlain *et al*. 1988; Bonhomme *et al*. 2005; Roeder *et al*. 2006). However, the number of microsatellite loci that can be multiplexed in a single reaction is limited by the number of usable primer dye-tags, as fragments from different loci of similar size ranges and tagged with the same dye will be difficult to distinguish from one another once analysed. Furthermore, considerable optimization may be necessary. Two-step multiplex PCR employs two successive PCRs. The first reaction contains primers from all loci under study and a limited number of cycles are performed. The second reaction uses diluted aliquots of the product from the first reaction as templates for individual singleplex PCRs at each locus. This method retains the advantage of one-step multiplex PCR in requiring very little original template DNA. However, the two-step multiplex PCR has the advantages of not requiring nonoverlapping fragment sizes and distinct dye-tags, because each locus is amplified individually in the second step. Furthermore, since two-step multiplexing initially targets all of the loci in the first reaction, less DNA extract is used than in most one-step multiplexing reactions, which requires several separate multiplexes when many loci are under investigation. Yet more important, is the reported increase in PCR amplification success rate and decrease in the frequencies of allelic dropout and false alleles (Piggot *et al*. 2004; Hedmark & Ellegren 2006; Lampa *et al*. 2008). However, these studies used few loci simultaneously (six: Bellemain & Taberlet 2004; Piggot *et al*. 2004; Lampa *et al*. 2008; five: Hedmark & Ellegren 2006), and no or non-quantitative measures of template DNA concentration. In addition, some protocols described thus far for two-step multiplex PCR typically suggest the use of large initial PCR volumes (50 μL, using 12 μL DNA template; Piggot *et al*. 2004; Hedmark & Ellegren 2006), and hence, are potentially costly in materials or require some optimization of primer combinations and/or development of internal 'nested' primers (Roempler *et al*. 2006; Lampa *et al*. 2008).

Here we present an easily adoptable two-step multiplex PCR protocol, which requires minimal optimization from standard singleplex PCR protocols, uses small amounts of template DNA (5 μL per PCR) and can incorporate at least 19 microsatellite primer pairs. The approach was extensively evaluated using faecal DNA from four different primate species (mountain gorillas: *Gorilla berengei berengei*; western gorillas: *Gorilla gorilla gorilla*; western chimpanzees: *Pan troglodytes verus*; and black and white colobus monkeys: *Colobus guereza*) and historical DNA from 100-year-old Cross River gorilla teeth (*Gorilla gorilla diehli*) from museum specimens. We ascertained the amount of DNA in each sample using a quantitative PCR assay (Morin *et al*. 2001) to establish the number of PCR replicates required to confidently type homozygous loci depending on the amount of template used. We show that the two-step multiplex PCR method, with little optimization, results in high PCR success rate and highly accurate genotypes even with very low concentration DNA templates, and that use of nested primers produces no discernible advantages.

Materials and methods

Sample collection, DNA extraction and quantification

Faecal samples from mountain gorillas, western lowland gorillas, western chimpanzees and black and white colobus monkeys were collected in Bwindi Impenetrable National Park, Uganda; Loango National Park, Gabon; Taï National Park, Côte d'Ivoire; and Kibale National Park, Uganda, respectively, using the two-step ethanol–silica storage method (Nsubuga *et al*. 2004) and were extracted using the QIAmp Stool kit (QIAGEN) with slight modifications (Nsubuga *et al*. 2004). Faecal samples were stored in the field for up to a year after collection and then at 4 °C thereafter. Samples were extracted from 3 months to 3 years after collection and storage. Approximately 100-year-old teeth from Cross River gorilla specimens from Cameroon and Nigeria were obtained from the Berlin Museum of Natural History, Germany and processed in an ancient DNA facility using a previously described alcohol precipitation extraction procedure (Vigilant *et al*. 2001). DNA quantification was perfomed using a 5′-nuclease assay targeting a highly conserved 81-bp portion of the c-myc proto-oncogene as described in Morin *et al*. (2001).

Microsatellite amplification and analysis

Using DNA from faeces, we amplified 16 microsatellite loci from mountain gorillas ($N = 343$ extracts), 15 from western

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

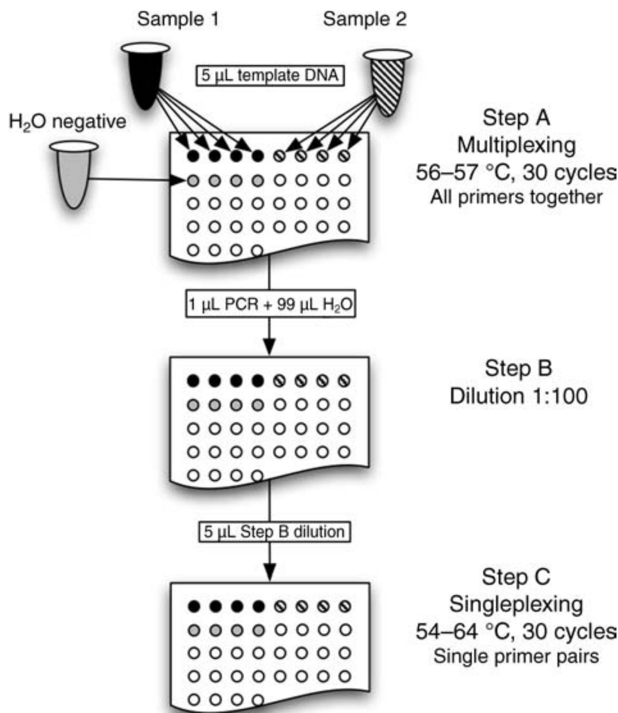

Fig. 1 Flowchart illustrating the steps involved in the two-step multiplex PCR set-up.

lowland gorillas (N = 229), 19 from western chimpanzees (N = 190) and 15 from black and white colobus (N = 165). Eight microsatellite loci were analysed in the Cross River gorilla museum specimens (N = 15). Nested reverse primers were designed for all loci with available sequences so that all 19 chimpanzee, 13 of 15 colobus and 8 of 16 gorilla loci were amplified with nested reverse primers to decrease the occurrence of primer dimers and increase PCR efficiency as suggested by Roempler *et al*. (2006) (Table S1, Supporting information).

In the initial multiplexing step, all microsatellite loci were amplified in a single reaction in 20-µL reaction volumes consisting of 1× Super*Taq* buffer (HT Biotechnology), 1.75 mm $MgCl_2$, 0.15 mm of each forward (unlabelled) and reverse (unnested) primer, 110 µm of each dNTP, 16 µg bovine serum albumin (BSA), 0.5 U Super*Taq* (HT Biotechnology) premixed 2:1 with *Taq*Start Antibody (BD Biosciences), and 5 µL template DNA. PCR thermocycling was performed in a PTC-200 thermocycler (MJ Research) with the following parameters as suggested in Roempler *et al*. (2006): initial denaturation for 9 min at 94 °C, 30 cycles of 20 s at 94 °C, 30 s at 55 °C, 56 °C or 57 °C (for chimpanzees, black and white colobus and gorillas, respectively), and 30 s at 72 °C, and a final extension of 4 min at 72 °C (Fig. 1). Initially, three to four independent amplifications of each sample were performed in 96-well plates, along with a minimum of five negative controls (where 5 µL H_2O rather than DNA was added to the well). Samples with ambiguous results (when one or more homozygote genotypes and a heterozygote genotype were amplified initially or samples with poor amplification success) were amplified in up to 12 replicates. Singleplex PCRs were carried out as above but with the following modifications: 5 µL of 1:100 diluted multiplex PCR product was used as template, half the amount of $MgCl_2$ (0.875 mm) was added, and only 0.35 U of Super*Taq* premixed 2:1 with *Taq*Start antibody was used. Furthermore, each singleplex PCR contained a single primer pair: 0.25 mm of a FAM, HEX or NED fluorescently labelled forward primer and 0.25 mm of a reverse primer, which was nested in some cases. Finally, the cycling conditions were as above except primer-specific annealing temperatures were used for each singleplex PCR and varied from 54 °C to 64 °C (Table S1).

To guard against contamination, all steps of the PCR set-up for faecal DNA samples (except the addition of template) were performed under a hood that was ultraviolet (UV) irradiated before and after use. When using the DNA from museum specimens, the initial addition of DNA to the multiplex PCR was carried out in a UV-irradiated ancient DNA laboratory in addition to the above precautions (Roempler *et al*. 2006).

Up to four different PCR products from the second singleplex amplification step were combined and electrophoresed on an ABI PRISM 3100 Genetic Analyser and alleles were sized relative to an internal size standard (ROX labelled HD400) using GeneMapper Software version 3.7 (Applied Biosystems).

PCR success, allelic dropout, and false allele amplification

For each extract, PCR success was calculated by dividing the number of PCRs that yielded an amplification product by the total number of PCRs attempted for all loci. PCR success was averaged across all extracts to obtain the overall PCR success rate. Only extracts that amplified at a minimum of three loci were included in the final data set, so that all results reflect PCR success using quantified template DNA and are not influenced by sample-specific problems with collection or extraction. This resulted in 343 of 421 (81%) mountain gorilla extracts, 229 of 310 (74%) western lowland gorilla extracts, 190 of 216 (88%) western chimpanzee extracts, 165 of 174 (95%) black and white colobus extracts and 15 of 19 (79%) Cross River gorilla samples being incorporated into the analyses.

To assess the dropout rate, the data set was first limited to extracts that yielded a heterozygote genotype at a given locus (heterozygotes were determined by observing each allele at least twice). Dropout was then calculated as the number of times only one of the two heterozygote alleles was amplified in a reaction, divided by the total number of PCRs when either or both alleles were seen. Averaging

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

across all extracts determined the overall dropout rate for any given locus.

Using the method of Taberlet *et al*. (1996), we assessed how many PCR replicates are necessary in order to achieve 99% certainty that a homozygote is indeed such at a given locus, and not a result of dropout at a heterozygous locus due to low template DNA quantity. This was carried out by assessing the dropout rate for each extract, categorizing extracts according to DNA template amount (measured in pg/5 μL) following the categories of Morin *et al*. (2001) and calculating the average dropout rate for these categories. For example, if the average dropout rate for extracts in a given DNA quantity category is 25%, then $(0.25)^4 < 0.01$, and thus four replicates are necessary to be 99% certain that apparently homozygous genotypes of extracts in this category are indeed homozygotes.

Average PCR success and the occurrence of allelic dropout with the two-step multiplex technique were analysed for each primate taxon separately and compared to the results from a similar data set that used one-step singleplex PCR (Morin *et al*. 2001). We also examined the relationship between the starting amount of template DNA used in the PCR, with PCR success and allelic dropout rates. To achieve robust results for all DNA template quantities, the data from all four species studied using faecal DNA were pooled, as some of the lower quantities of DNA were underrepresented in each data set alone. For example, there were only 27 mountain gorilla, 7 western lowland gorilla, 4 western chimpanzee and 7 black and white colobus extracts that contained less than 5 pg/μL DNA [25 pg/reaction (rxn)].

Based on the criteria above, homozygotes and heterozygotes were scored with 99% certainty, thus making it possible to identify the occurrence of false alleles. False alleles were designated as peaks bearing allele sizes that were within or one repeat unit away from the allele size ranges in the population for a locus, and were not confirmed in subsequent PCRs. The overall false allele rate for any given locus was calculated by summing the number of times false alleles were seen in all samples for each locus, and then averaging across samples.

The allelic dropout error rate and number of replicates required calculated herein only accounts for cases when every replicate gives a homozygote genotype for a given sample and locus. When it is observed that one replicate gives a heterozygote genotype and one or more replicates give homozygote genotypes, then additional PCRs should be undertaken and equation 3 of Puechmaille & Petit (2007) applied. If, for example, the average dropout rate for a given DNA quantity category is 25%, then $(0.25)^6 + 6X(0.25)^{6-1}X(1-0.25) < 0.01$, and so six replicates yielding a homozygous result are necessary to be 99% certain that the sample is indeed a homozygote and that an aberrant allele seen once can be considered a false allele. When two or more of the additional replicates give heterozygous genotypes, the sample should be considered heterozygous for that locus.

Nested and unnested primers

To evaluate whether nested primers performed better than unnested primers, we compare gorilla samples amplified using nested and unnested primers for different loci using 2-tailed exact Mann–Whitney *U*-tests as we calculated the success and dropout rates for two different sets of loci. Although we do not compare the success rates for the two primer types for the same loci, nested primers were only designed for loci with available sequence data and not according to success rate data, or amplifiable fragment size. As such, we have no reason to suspect that ascertainment bias might influence the results of this comparison.

Results

PCR success, allelic dropout, and false allele amplification

Primate faecal sample DNAs had high levels of PCR success (90–94%), and low occurrences of allelic dropout (4–9%) and false alleles (0.27–1.72%) (Table 1). PCR success from Cross River gorilla museum specimen DNAs was on average 74%, allelic dropout was 42%, and the occurrence of false allele rate was 0.80%. Nearly all of the DNA extracts from museum specimens (13 of 15) contained less than 1 pg/μL of DNA, and the other two extracts contained 2 pg/μL and 5 pg/μL. In contrast, faecal DNA extracts were estimated using quantitative PCR to contain from < 1 pg/μL to > 1000 pg/μL.

As expected, allelic dropout and PCR failure were most often observed at the lowest DNA template amounts and quickly improved as the amount of DNA in the PCR increased (Fig. 2, Fig. S1, Supporting information). However, even amplifications using the smallest amount of template (≤ 25 pg) produced a PCR product in 70% of attempts.

Based on the allelic dropout values obtained for each of the categories, five replicates, calculated as $(0.32)^5$ are needed to reach 99% certainty for the ≤ 25 pg of template DNA category, four are necessary for the 26–50 pg/rxn category, three for reactions containing 51–100 pg of template and two for template amounts of ≥ 101 pg (Table 2). When the number of replicates required according to template amount were calculated by species (Table S2, Supporting information), the mountain gorilla data set followed the replicate rules cited above. The western lowland gorillas, western chimpanzees and black and white colobus data sets differed in that they required only four replicates for the ≤ 25 pg of template DNA category and that the black and white colobus data set suggested only three replicates for the 26–50 pg category. Thus, the consensus rules from

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

Table 1 Average success, dropout and false allele rates of the two-step multiplex PCR system for DNA obtained from low-quantity DNA sources for five primate taxa

Species (sample source)	No. of PCRs	No. of loci	Mean DNA amount ± SD (pg/μL)*	PCR success	Allelic dropout†	False alleles
Mountain gorilla (faeces)	15950	16	149 ± 329 (0–4431)	91%	6% (9756)	0.27%
Western lowland gorilla (faeces)	11007	15	241 ± 593 (0–6921)	94%	9% (7408)	1.72%
Western chimpanzee (faeces)	9863	19	454 ± 975 (3–10258)	92%	4% (6566)	0.38%
Black and white colobus (faeces)	8430	15	387 ± 996 (0–6908)	90%	4% (3843)	< 0.50%
Cross River gorilla (~100 year old teeth)	1121	8	< 1 (0–5)	74%	42% (427)	0.80%
One-step singleplex PCR (Morin *et al*. 2001) Western chimpanzee (faeces)	1800	9	192 (0–2550)	79%	24% (1300)	< 1.00%

*SD, standard deviation (values in parentheses represent the minimum and maximum DNA quantities obtained); †total number of reactions that amplified one or both alleles of a heterozygote genotype given in parentheses.

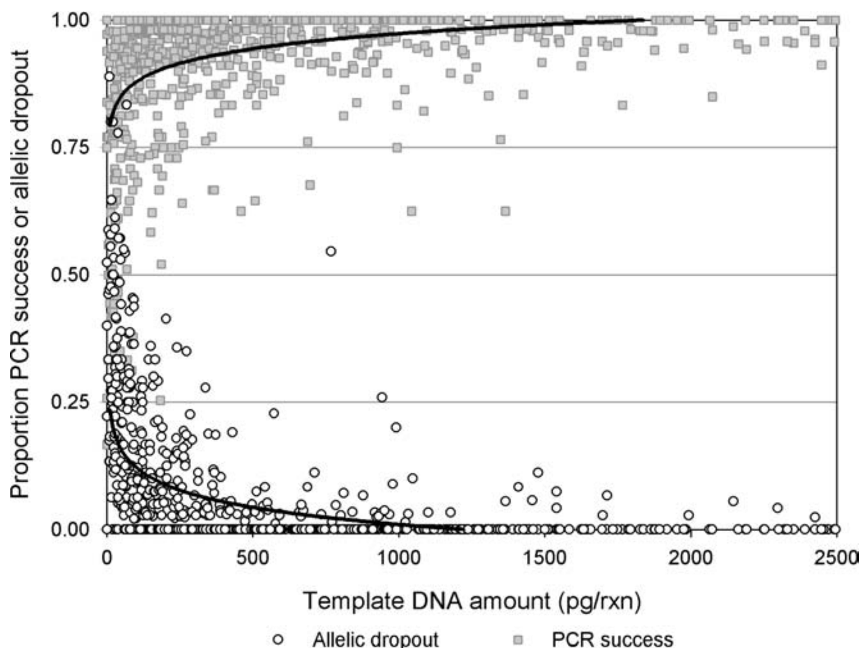

Fig. 2 Proportion of successful PCRs (grey squares) and PCRs with allelic dropout (white circles) plotted according to initial template DNA amount in the PCR. Each point represents one faecal DNA extract from either mountain gorillas, western lowland gorillas, western chimpanzees or black and white colobus. On average, 49 amplifications, 30 of which produced heterozygote genotypes, were attempted per sample. The PCR success data produce a curve with the equation $y = 0.7162x^{0.0444}$, while the allelic dropout data are best described by the curve $y = 0.3466–0.0488 \ln(x)$. Data from 0 to 2500 pg/rxn DNA shown only.

the pooled data sets are conservative. As all but two of the Cross River gorilla museum specimen DNAs contained < 1 pg/μL DNA, these samples require one more replicate (six) to achieve 99% certainty in homozygote genotypes.

In cases where DNA extracts were from particularly essential study animals but did not amplify well with the above-described technique, two modifications proved to be beneficial (data not shown). First, dilution of extracts up to 1:10, to reduce the effect of potential inhibitors reported to exist in faeces (Monteiro *et al*. 1997), was beneficial for a small proportion of samples. When this did not achieve consistent results, we obtained improved results for ~75% of samples with previously low success rates by initially amplifying DNA in 60-μL reaction volumes and tripling all the reagents accordingly.

Nested and unnested primers

When compared in the two gorilla data sets, nested and unnested primers did not differ in their PCR success or allelic dropout rates (success rate: mountain gorillas, Mann–

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

Whitney $U = 26$, $N_{nested} = 8$, $N_{unnested} = 8$, $P = 0.574$; western lowland gorillas, Mann–Whitney $U = 24$, $N_{nested} = 8$, $N_{unnested} = 7$, $P = 0.694$. Dropout rate: mountain gorillas, Mann–Whitney $U = 22$, $N_{nested} = 8$, $N_{unnested} = 8$, $P = 0.328$; western lowland gorillas, Mann–Whitney $U = 14$, $N_{nested} = 8$, $N_{unnested} = 7$, $P = 0.121$) (Table S3, Supporting information).

Discussion

Amplification success and allelic dropout rates obtained using the two-step multiplex PCR approach show an improvement over traditional one-step PCRs. Average PCR success rates ranged from 90% to 94%, reflecting a 10–14% increase in PCR success compared to the singleplex method used in Morin *et al*. (2001), and similar to the results found in other two-step multiplex studies (75–88%: Piggot *et al*. 2004; 91%: Hedmark & Ellegren 2006; 78%: Lampa *et al*. 2008). Allelic dropout ranged from 4% to 9%, representing a 15% to 20% decrease when using the two-step multiplex PCR approach over the results reported in Morin *et al*. (2001). These allelic dropout rates are comparable or better to those reported in some other two-step multiplex PCR papers (2.4%: Hedmark & Ellegren 2006; median 29%: Lampa *et al*. 2008) but poorer than those reported in Piggot *et al*. (2004) (0–0.21%). False alleles occurred with an average frequency of 0.27% to 1.72%, which is similar to, or slightly higher than, other PCR systems (< 1%: Morin *et al*. 2001; 0–0.02%: Piggot *et al*. 2004; 0.4%: Hedmark & Ellegren 2006; 0.02%: Lampa *et al*. 2008). However, as their occurrence is still relatively low and readily detected through PCR replication, we assume as in other studies, the false allele rate should be a minor problem with this two-step multiplex PCR system (Sloane *et al*. 2000; Hedmark *et al*. 2004).

It is important to note that we use different samples, preservation methods, loci and PCR reagents in our two-step multiplex study than those used in Morin *et al*. (2001), Piggot *et al*. (2004), Hedmark & Ellegren (2006) and Lampa *et al*. (2008). Some studies have shown that even when preservation and PCR conditions are held constant, samples can exhibit up to fivefold worse dropout rates (Puechmaille *et al*. 2007). As we quantified the amount of DNA in our samples however, we can be assured that the samples in our study and those in Morin *et al*. (2001) are comparable, at least in terms of DNA quantity. Without more extensive testing, however, it is difficult to show that the two-step multiplex PCR approach is the factor underlying the observed improvements over singleplex PCR as outlined in Morin *et al*. (2001). Nevertheless, we have several arguments that suggest that this is the case. First, the improvement in PCR efficiency when using the two-step multiplex approach is most probably attributed to an improvement in amplification technique and not due to an overall increase in DNA quantity (due to better preservation techniques and/or by a larger reaction volume of template

Table 2 Number of PCR replicates necessary for various categories of initial DNA template amount, to assure with high confidence (> 99% certainty) that homozygote genotypes are authentic and not the result of allelic dropout

	Two-step multiplex PCR (this study)						One-step singleplex PCR (Morin *et al*. 2001)	
	Primate faecal samples			Cross River gorilla 100-year-old teeth			Western chimpanzee	
Template DNA amount (pg/rxn)	PCR success*	Allelic dropout†	Reps needed	PCR Success*	Allelic dropout†	Reps needed	Allelic dropout†	Reps needed
≤ 25	68% (2168)	32% (941)	5	74% (1121)	42% (421)	6	68% (119)	Do not use
26–50	79% (2594)	27% (1219)	4				42% (369)‡	7
51–100	87% (3787)	17% (2107)	3				42% (369)‡	7
101–200	92% (6525)	7% (3999)	2				26% (184)	4
≥ 201	96% (30176)	2% (19307)	2				5.2% (656)	2

*Total number of analysed PCRs given in parentheses. Success by template DNA amount category, not reported in Morin *et al*. (2001); †total number of analysed PCRs that amplified one or both alleles of a heterozygote genotype given in parentheses; ‡category of 26–100 reported in Morin *et al*. (2001).

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

DNA), since equivalently low concentration of faecal and historical template DNA are amplified more successfully and with less dropout than in the study by Morin *et al*. (2001) (Table 2). In fact, Morin *et al*. (2001) advised against using our smallest category of template DNA (< 25 pg/rxn) due to the then observed high frequency of dropout (68%) and the consequent number of replicates theoretically necessary (12) to achieve reliable results. Thus, before the validation of this multiplex approach, the Cross River gorilla museum samples could only have been reliably genotyped with considerable effort. With the two-step multiplex PCR technique, all extracts that yield products at three loci or more, including those containing very small amounts of DNA, are usable and require fewer PCR replicates than before. Second, it is possible that the ethanol preservation step now used in the two-step sample collection reduces inhibitors in the samples and it is this factor which is the underlying cause of the PCR improvement seen with the two-step method. However, we suggest that this is not the case as the DNA quantification assay is designed so that if a sample is inhibited, the quantity of DNA detected by the assay is low. Finally, our laboratory has used samples collected with the two-step method using the singleplex PCR method and the general consensus is that samples that contained low quantities of DNA still amplified poorly. The same can be said for the use of two different *Taq* polymerases in this and the Morin *et al*. (2001) study.

The utility of DNA quantification for increasing and streamlining PCR efficiency was demonstrated by Morin *et al*. (2001) and is re-emphasized by this study. If the concentration of DNA template is known, it is possible to conduct duplicate PCRs on every sample initially, and only if there is PCR failure or if a potentially homozygous sample contains less than 20 pg/μL DNA are further PCRs required (up to three more, for samples with the least amount of DNA). If DNA quantification is not undertaken, the only conservative way to proceed is to genotype all potential homozygotes five times – a much more inefficient strategy, particularly for large sample sizes. Our study stands in contrast to previous reports which suggested (without DNA quantification of their samples) that fewer than five replicates are sufficient to produce reliable genotypes (Banks *et al*. 2002; Bellemain *et al*. 2005; Piggott *et al*. 2006). Here we have shown that without DNA quantification, the minimum number of replicates to perform is five based on the four taxa we investigated, and this implies that use of fewer replicates may lead to unacceptable levels of error.

In general, the two-step multiplex PCR protocol presented here uses less sample DNA when compared to other described approaches, as DNA is only added to the initial PCR replicates and not to several multiplex or singleplex PCRs. For example, a traditional one-step singleplex PCR, as in Morin *et al*. (2001), uses 2 μL DNA extract for each of two to seven independent PCRs per locus. Therefore, for 10 loci this would require from 40 to 140 μL of DNA extract. Using the two-step multiplex approach, 5 μL DNA extract for each of two to five independent PCRs are required, resulting here in the use of 10 to 25 μL DNA extract for 19 (and potentially even more) loci. This example assumes that every PCR is successful. Yet the two-step is more successful than the one-step system, so in effect, the difference in efficiency is probably even greater. The two-step method is also an improvement over the one-step PCR approach in that it is only the initial multiplex PCR step that is loaded with DNA well by well. All subsequent singleplex PCRs are loaded in tandem with multichannel pipettes saving not only template DNA, but also loading time and effort while also reducing potential human error.

To our knowledge, one-step multiplexing has not been shown to improve success, dropout or false allele rates in comparison to one-step singleplex PCR. As such, the improvements seen with the two-step approach here are expected also when comparing this technique to that of one-step multiplexing. It must be noted however, that the two-step method is more time-consuming than one-step multiplexing, especially if few loci are analysed. If one-step multiplex success rates are comparable or only slightly worse than those of the two-step multiplex technique, then the two-step technique is also more costly in terms of reagents. It is however, our experience that the two-step multiplex method greatly improves the success, dropout and false allele rates and results in fewer replicates needed to ensure genotypes with high confidence overall. Furthermore, because the technique involves two-steps, the chance of contamination is higher than with traditional techniques, we have found however, that when PCRs are set-up in dedicated UV-irradiated hoods, very little contamination occurs.

The two-step multiplex PCR protocol reported here also has several benefits over other pre-amplification PCR systems. First, we were able to amplify up to 19 loci simultaneously, which is considerably more than the five or six microsatellite loci previously described (Piggot *et al*. 2004; Hedmark & Ellegren 2006; Lampa *et al*. 2008) and probably does not represent the upper limit of number of primers that can be combined. In fact, more than 40 primer pairs have been successfully used to amplify ancient DNA fragments using a similar two-step multiplex technique, and with higher quality DNA even more primer pairs are possible (Römpler *et al*. 2006). Although we had limited success in amplifying more than one locus at a time in the second step of the procedure (data not shown), we did not exhaustively try to optimize the PCR conditions to do so successfully. As multiplexing the second step of the system has been carried out with success in other studies (Bellemain & Taberlet 2004; Hedmark & Ellegren 2006; Lampa *et al*. 2008), doing so remains a possibility for those who wish to further reduce the time involved in this technique.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

We were also able to use primers with estimated annealing temperatures differing by up to 10 °C together in the first multiplex step by running the multiplex reaction at an intermediate annealing temperature (55 °C, 56 °C or 57 °C depending on the set of loci) during the multiplex step, and then at their estimated ideal temperatures during the singleplex step. The successful use of an intermediate annealing temperature to amplify all loci is a considerable benefit of the technique as it removes a large hurdle often associated with multiplexing; the optimization of compatible primers with respect to their annealing temperatures, amplicon size ranges and dye-tags. In fact, our system required very little optimization in the first step (increasing the amount of $MgCl_2$ and uniformly decreasing the primer concentrations) and second step (decreasing the amount of polymerase) when compared to our standard one-step singleplex protocol, making this method easily transferable to other laboratories. Adding to this is the suggestion herein that nesting primers does not improve PCR success nor decrease allelic dropout as suggested by Römpler *et al*. (2006). Thus, researchers can use the same primer sets they currently employ and simply obtain unlabelled primers, if this two-step multiplex PCR technique is to be adopted.

Conclusion

The two-step multiplexing PCR system allows rapid, efficient and reliable genotyping of high numbers of samples from low-quantity DNA sources such as faeces and historical museum specimens. The method allows for many loci to be amplified simultaneously, saving valuable samples and processing time. In comparison to singleplex PCR techniques, samples that would have been excluded from the analysis before can now be used and fewer PCR replicates are now required to achieve the same high levels of genotype reliability. Furthermore, the technique should easily be adaptable to protocols currently used in most laboratories, as primers do not need to be re-designed and the reagents only need to be slightly adjusted from current running procedures. When combined with DNA quantification, the technique can potentially save a great deal of time and materials by requiring fewer PCR replicates for samples with moderate to high quantities of DNA.

Acknowledgements

We thank P. Taberlet for helpful discussions, A. Abraham, T. Fuenfstuck, S. Hinrich, C. Lang, F. Schildhauer & M. Spitzner for laboratory assistance, R. Mundry for statistical assistance and four anonymous reviewers for valuable comments on the manuscript. We also thank the Uganda Wildlife Authority, Uganda National Council for Science and Technology, Conseil National des Parcs Nationaux of Gabon, the Ivorian Ministry of the Environment and Forests, the Ivorian Ministry of Research and the Centre Suisse de Recherches Scientifiques in Abidjan, Ivory Coast. This work was funded by the Max Planck Society, the Humboldt Foundation, the Societe pour la Conservation et le Developpement and the Wildlife Conservation Society.

References

Ball MC, Pither R, Manseau M *et al*. (2007) Characterization of *target* nuclear DNA from faeces reduces technical issues associated with the assumption of low-quality and quantity template. *Conservation Genetics*, **8**, 577–586.

Banks SC, Piggott MP, Hansen BD, Robinson NA, Taylor AC (2002) Wombat coprogenetics: enumerating a common wombat population by microsatellite analysis of faecal DNA. *Australian Journal of Zoology*, **50**, 193–204.

Bayes MK, Smith KL, Alberts SC, Altman J, Bruford MW (2000) Testing the reliability of microsatellite typing from fecal DNA in the savannah baboon. *Conservation Genetics*, **1**, 173–176.

Bellemain E, Taberlet P (2004) Improved noninvasive genotyping method: application to brown bear (*Ursus arctos)* faeces. *Molecular Ecology Notes*, **4**, 519–522.

Bellemain E, Swenson JE, Tallmon D, Brunberg S, Taberlet P (2005) Estimating population size of elusive animals with DNA from hunter-collected feces: four methods for brown bears. *Conservation Biology*, **19**, 150–161.

Bonhomme M, Blancher A, Crouau-Roy B (2005) Mutiplexed microsatellites for rapid identification and characterization of individuals and populations of Cercopithecidae. *American Journal of Primatology*, **67**, 385–391.

Broquet T, Petit E (2004) Quantifying genotyping errors in non-invasive population genetics. *Molecular Ecology*, **13**, 3601–3608.

Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN, Caskey CT (1988) Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. *Nucleic Acids Research*, **16**, 11141–11156.

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

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

Hansen H, Ben-David M, McDonald DB (2008) Effects of genotyping protocols on success and errors in identifying individual river otters (*Lontra Canadensis*) from their feces. *Molecular Ecology Resources*, **8**, 282–289.

Hedmark E, Ellegren H (2006) A test of the multiplex pre-amplification approach in microsatellite genotyping of wolverine faecal DNA. *Conservation Genetics*, **7**, 289–293.

Hedmark E, Flagstad O, Segerström P *et al*. (2004) DNA-based individual and sex identification from wolverine (*Gulo Gulo*) faeces and urine. *Conservation Genetics*, **5**, 405–410.

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

Lampa S, Gruber B, Henle K, Hoehn M (2008) An optimisation approach to increase DNA amplification success of otter faeces. *Conservation Genetics*, **9**, 201–210.

Miquel C, Bellemain E, Poillot C *et al*. (2006) Quality indexes to assess the reliability of genotypes in studies using noninvasive sampling and multiple-tube approach. *Molecular Ecology Notes*, **6**, 985–988.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd

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, Chambers KE, Boesch C, Vigilant L (2001) Quantitative PCR analysis of DNA from noninvasive samples for accurate microsatellite genotyping of wild chimpanzees (*Pan troglodytes verus*). *Molecular Ecology*, **10**, 1835–1844.

Murphy MA, Waits LP, Kendall KC *et al*. (2002) An evaluation of long-term preservation methods for brown bear (*Ursus arctos*) faecal DNA samples. *Conservation Genetics*, **3**, 435–440.

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.

Nsubuga AM, Robbins MM, Roeder A *et al*. (2004) Factors affecting the amount of genomic DNA extracted from ape feces and the identification of an improved sample storage method. *Molecular Ecology*, **13**, 2089–2094.

Piggot MP, Bellemain E, Taberlet P, Taylor AC (2004) A multiplex pre-amplification method that significantly improves microsatellite amplification and error rate for faecal DNA in limiting conditions. *Conservation Genetics*, **5**, 417–420.

Piggott MP, Banks SC, Stone N, Banffy C, Taylor AC (2006) Estimating population size of endangered brush-tailed rock-wallaby (*Petrogale penicillata*) colonies using faecal DNA. *Molecular Ecology*, **15**, 81–91.

Puechmaille SJ, Petit EJ (2007) Empirical evaluation of non-invasive capture-mark-recpature estimation of population size based on a single sampling session. *Journal of Applied Ecology*, **44**, 843–852.

Puechmaille SJ, Mathy G, Petit EJ (2007) Good DNA from bat droppings. *Acta Chiropterologica*, **9**, 269–276.

Roeder AD, Archer FI, Poinar HN, Morin PA (2004) A novel method for collection and preservation of faeces for genetic studies. *Molecular Ecology Notes*, **4**, 761–764.

Roeder AD, Jeffery K, Bruford MW, the INPRIMAT consortium (2006) A universal microsatellite multiplex kit for genetic analysis of great apes. *Folia Primatologica*, **77**, 240–245.

Roempler H, Dear PH, Krause J *et al*. (2006) Multiplex amplification of ancient DNA. *Nature Protocols*, **1**, 720–728.

Roon DA, Waits LP, Kendall KC (2003) A quantitative evaluation of two methods for preserving hair samples. *Molecular Ecology Notes*, **3**, 163–166.

Sloane MA, Sunnucks P, Alpers D, Beheregaray B, Taylor AC (2000) Highly reliable genetic identification of individual northern hairy-nosed wombats from single remotely collected hairs: a feasible censusing method. *Molecular Ecology*, **9**, 1233–1240.

Taberlet P, Griffin S, Goossens B *et al*. (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research*, **26**, 3189–3194.

Taberlet P, Waits LP, Luikart G (1999) Noninvasive genetic sampling: look before you leap. *Trends in Ecology & Evolution*, **14**, 321–325.

Vigilant L, Hofreiter M, Siedel H, Boesch C (2001) Paternity and relatedness in wild chimpanzee communities. *Proceedings of the National Academy of Sciences, USA*, **98**, 12890–12895.

Wehausen JD, Ramey RR, Epps CW (2004) Experiments in DNA extraction and PCR amplification from bighorn sheep feces: the importance of DNA extraction method. *Journal of Heredity*, **95**, 503–509.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 Proportion of successful PCRs (black circles) and PCRs with allelic dropout (white circles) according to initial template DNA amount in the PCR, for four primate taxa. (a) Mountain gorillas (b) western lowland gorillas (c) western chimpanzees and (d) black and white colobus. Data from 0 to 2500 pg/rxn DNA shown only.

Table S1 Primers used in study

Table S2 Number of PCR replicates necessary for various categories of initial DNA template amount, to ensure with high confidence (> 99% certainty) that homozygote genotypes are authentic and not the result of allelic dropout, for each primate taxa

Table S3 Nested and unnested primers do not differ in their PCR success or allelic dropout rates

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd