Nucleic Acids Research, 1996, Vol. 24, No. 16 **3189–3194**

Reliable genotyping of samples with very low DNA quantities using PCR

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Received May 1, 1996; Revised and Accepted July 2, 1996

ABSTRACT

Our purpose was to identify an experimental procedure using PCR that provides a reliable genotype at a microsatellite locus using only a few picograms of template DNA. Under these circumstances, it is possible (i) that one allele of a heterozygous individual will not be detected and (ii) that PCR-generated alleles or 'false alleles' will arise. A mathematical model has been developed to account for stochastic events when pipetting template DNA in a very dilute DNA extract and computer simulations have been performed. Laboratory experiments were also carried out using DNA extracted from a bear feces sample to determine if experimental results correlate with the mathematical model. The results of 150 typing experiments are consistent with the proposed model. Based on this model and the level of observed false alleles, an experimental procedure using the multiple tubes approach is proposed to obtain reliable genotypes with a confidence level of 99%. This multiple tubes procedure should be systematically used when genotyping nuclear loci of ancient or forensic samples, museum specimens and hair or feces of free ranging animals.

INTRODUCTION

The polymerase chain reaction (PCR) (1–3) is an enzymatic process by which a specific region of DNA is replicated repeatedly to yield several million copies of a particular sequence. Typically, nanogram to microgram quantities of genomic DNA are used as a template for the PCR. This corresponds to ~300–300 000 copies of a unique target sequence. Refinements of the standard technique have even enabled the amplification of DNA extracted from a single spermatozoid (see for example 4–6), demonstrating that a single molecule can be reliably amplified (7). In order to improve the sensitivity of the reaction when the template DNA contains very few copies of the target sequence, a two-step PCR can be performed (8). Because of this extreme sensitivity, there is a high risk of contamination, against which strict measures must be taken (see for example 9,10).

In molecular studies using ancient samples, forensic samples, museum specimens and hair or feces of free ranging animals, the amount of DNA available for genetic typing can be very low and is often in the picogram range. Under these circumstances, it is possible for one allele of a heterozygous individual not to be detected, leading to an incorrect genotyping of this individual as a homozygote. This kind of problem has been encountered in a microsatellite study using Bonobo (*Pan pygmeus*) feces (11). A theoretical study by Navidi *et al.* (12) suggested that the use of a multiple tubes approach, distributing the extract DNA among several tubes, provides more reliable genotyping of dilute DNA samples than a single tube approach. To our knowledge, this has not been tested experimentally.

In order to identify each individual of the endangered Pyrenean brown bear population (*Ursus arctos*), we are currently studying microsatellite polymorphism of DNA extracted from hairs and feces collected in the field (13,14). Our preliminary results clearly demonstrated that the amount of extracted DNA varies greatly among samples and that an incorrect conclusion of homozygosity may be reached for some extracts. We therefore decided to use the multiple tubes approach (12). Our basic purpose was to determine how many times an experiment would have to be repeated in order to obtain a reliable genetic typing of a homozygous locus, with the constraint that only a limited amount of template DNA is available. So, the greater the number of PCRs, the fewer the number of loci that can be analysed.

Furthermore, we initially obtained many more genotypes than expected, with each additional genotype corresponding to a previously observed genotype but with a single difference: one extra allele at an otherwise homozygous locus. We tried repeating experiments in order to confirm the presence of these alleles, but they were not reproduced, even with six or seven repetitions. The problem became even more complex when three alleles were obtained at the same locus for some heterozygous individuals. One possible explanation for these unusual results is sporadic contamination, either by cross-contamination between extracts or by PCR product carry-over. However, in our case, the contamination hypothesis can be ruled out. First, the alleles concerned were always obtained for the first time in our laboratory, therefore, PCR product carry-over cannot be responsible. Second, all of the bear DNA samples currently in the laboratory have been analysed

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and do not carry the alleles concerned, ruling out the possibility of cross-contamination. As an alternative hypothesis, we suggest that slippage artifacts generated during the first cycles of the PCR may explain these false alleles.

In this paper, we present an experimental procedure which provides reliable genotyping using very low amounts of template DNA, taking into account (i) the possibility of not detecting an allele in heterozygous individuals and (ii) the problem of PCR-generated false alleles. Such procedures should be applied when a few picograms of DNA are to be amplified to a detectable level, as is the case in studies of forensic samples, museum specimens and fossils.

MATERIALS AND METHODS

General strategy

Our strategy consisted of developing a mathematical model which takes into account stochastic events when pipetting template DNA in a very dilute DNA extract. Laboratory experiments were carried out in order to: (i) determine if experimental results correlate with the mathematical model; (ii) estimate the frequency of PCR-generated artifacts. Based on the results obtained, an appropriate experimental procedure was designed.

Mathematical model and computer simulation

The model is restricted to the genotyping of an individual bearing alleles A and B at an autosomal locus. The main purpose of the model is to take into account the stochastic sampling of DNA molecules when pipetting the template DNA for each PCR. The following assumptions have been made: (i) the DNA extract contains equal numbers of the alleles A and B; (ii) a single target molecule can be amplified and detected; (iii) each single target molecule has the same probability of being amplified; (iv) 100 PCRs can be performed using the DNA extract and the target DNA molecules are distributed randomly among the 100 PCR tubes; (v) if the initial proportion between alleles A and B (A/B or B/A) in the PCR tube is ≥ 5, then only the most common allele will be detected.

Assumption (iii) is related to previous studies concerning PCR on single spermatozoids that have shown that only ~60–70% of each DNA molecule can be amplified (4–6), so only amplifiable DNA is considered later. Assumption (v) is included because it is difficult to score an allele when the band intensity on the autoradiograph is five times weaker than the other allele (12). Other proportions were also tested, but the results were not substantially different.

The computer simulations were set up to randomly distribute the given amount of extracted DNA (i.e. the given number of alleles A and B) among 100 PCR tubes and then to analyse the results of the DNA amplification in each tube using the above assumptions. One thousand replications of random sampling of alleles without replacement were performed for 48 extracts of different DNA concentrations. The amount of amplifiable DNA in the extracts ranged from one to 1000 diploid genomes, corresponding to 0.01–10 diploid genomes/PCR tube.

Laboratory procedures

As a precaution against contamination, the extraction procedure was performed in a special room dedicated only to DNA extraction from ancient samples, hair and feces. In order to produce enough template DNA for the subsequent amplification, five DNA extractions were carried out at the same time for a single feces sample of a brown bear. This sample was collected in the field by J.-J.Camarra in the Pyrenees mountains. Extraction was performed using the silica method (15–17). The bear feces sample was preserved dry until DNA extraction.

For each extraction, ~50 mg dry bear feces was added to 1 ml L6 extraction buffer (10 M GuSCN, 0.1 M Tris–HCl, pH 6.4, 0.02 M EDTA, pH 8.0, 1.3% Triton X-100). After incubation overnight at 60°C with constant agitation, 500 µl of the liquid phase was added to 500 µl fresh L6 extraction buffer and 40µl silica suspension prepared as previously described (17). The mixture was incubated at room temperature for 10 min with constant agitation. After centrifugation (1 min, 7000 *g*), the silica pellet was washed three times with 500 µl L2 buffer (10 M GuSCN, 0.1 M Tris–HCl, pH 6.4), once with 1 ml 100% ethanol and once with 1 ml acetone. The pellet was then dried at 60°C for 10 min and nucleic acids were eluted at 60°C for 5 min with 200 µl TE (10 mM Tris–HCl, 10 mM EDTA, pH 8.3). The tube was centrifuged (3 min, 10 000 *g*) and 160 µl supernatant was carefully removed to avoid pipetting silica particles and transferred to a new Eppendorf tube. The tube was centrifuged again (3 min, 10 000 *g*) and only 120 µl was removed to ensure that no silica particles remained in the extract. The products of the five extractions were then pooled, constituting the 1:1 extract. Two aliquots of this 1:1 extract were used to make two extract dilutions of 1:2 and 1:4. Five microlitres of each extract was used as template for each PCR. To detect whether contamination of samples with exogenous DNA had occurred during the extraction procedure, a tube without bear feces (extraction negative control) was treated identically through both the extraction procedure and the amplification.

Fifty PCRs were carried out for each of the 1:1, 1:2 and 1:4 extracts. The DNA amplifications were performed in a two-step PCR. The first step used diluted external microsatellite primers to reduce the formation of primer–dimer artifacts (8). In the second step, a nested primer was introduced. Three primers were designed from the sequence of locus G10B (18): G10BF (5′-AAGCCTTTTAATGTTCTGTTG-3′); G10BFI (5′-TGCTA-ATATTTTCTTGAGGACT-3′); G10BR (5′-AGGACAAATCAC-AGAAACCT-3′. The first step was performed in a total volume of 25 µl [750 mM Tris–HCl, pH 9.0, 200 mM $(NH_4)_2SO_4$, 50 µM each dNTP, 1.5 mM $MgCl_2$, 5 ng BSA, 0.1 U Red GoldStar DNA polymerase (Eurogentec), 0.01 µM primer G10BF, 0.01 µM primer G10BR, 5 µl extract] and a PCR amplification of 20 cycles was carried out (93°C for 30 s, 55°C for 30 s, 72°C for 1 min, using a Perkin Elmer Gene Amp PCR System 9600). Between the first and second steps, a volume of 25 µl [750 mM Tris–HCl, pH 9.0, 200 mM $(NH_4)_2SO_4$, 50 µM each dNTP, 1.5 mM $MgCl_2$, 5 ng BSA, 0.1 U Red GoldStar DNA polymerase, 1 µM primer G10BFI and 1 µM primer G10BR] was added to the same tube. The second step consisted of 35 cycles of amplification (93°C for 30 s, 55°C for 30 s, 72°C for 1 min). The PCR products were purified on a low melting point agarose gel, diluted in 200 µl ddH_2O and 10 µl were used as template for an additional amplification of two cycles (93°C 10 s, 55°C 30 s, 72°C 1 min) performed in a volume of 25 µl [750 mM Tris–HCl, pH 9.0, 200 mM $(NH_4)_2SO_4$, 50 µM each dNTP, 1.5 mM $MgCl_2$, 0.1 U Red GoldStar DNA polymerase, 0.2 µM primer G10BF and 0.02 µM γ-^{33}P-labelled primer G10RI]. Amplification products

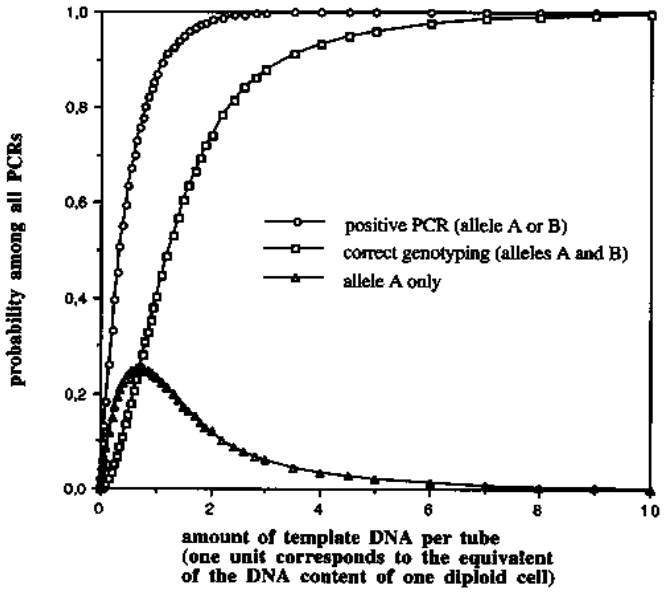

Figure 1. Results of the simulation concerning the genetic typing of a heterozygous individual bearing alleles A and B. Probability among all PCRs of obtaining a positive PCR (allele A or B), a correct genotype (alleles A and B) or only one allele (allele A only), according to the amount of template DNA.

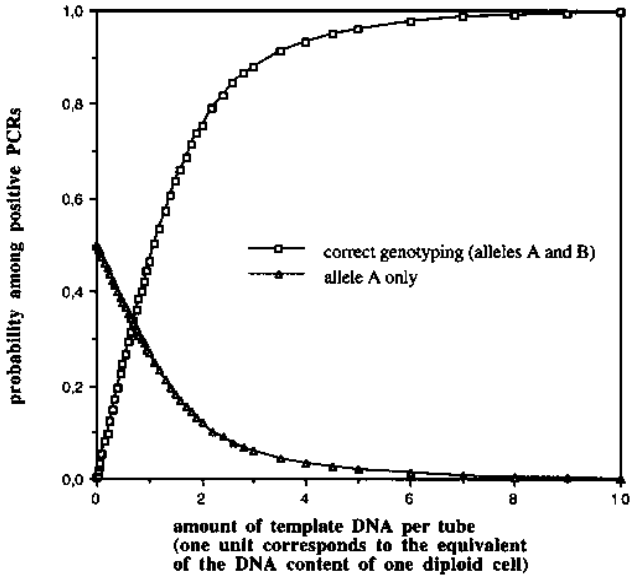

Figure 2. Results of the simulation concerning the genetic typing of a heterozygous individual bearing alleles A and B. Probability among positive PCRs of obtaining a correct genotyping (alleles A and B) or only one allele (allele A only), according to the amount of template DNA.

were separated by electrophoresis on a 6% polyacrylamide gel (sequencing gel) for 2 h. This gel was dried and exposed to autoradiography film.

RESULTS

Simulation

The stochastic sampling of target DNA in very dilute extracts is illustrated by a subset of the simulation results shown in Table 1. The number of target alleles varies greatly among tubes and some tubes do not contain any target molecules. Figure 1 shows the probabilities of obtaining a positive PCR (either allele A or B), only allele A or both alleles (correct genotype) when using different quantities of template DNA. One unit (U) of the template DNA corresponds to the amplifiable diploid content of one cell. For 1.5 U template DNA, the probability of obtaining a PCR product is 0.95. For 2.4 U template DNA, the probability of obtaining a PCR product is 0.99, but the probability of obtaining a correct genotype is only 0.80. The probabilities of identifying the correct genotype at the 0.95 and 0.99 levels are obtained for 5.0 and 8.0 U template DNA respectively. The maximum probability of obtaining only one allele is for 0.7 U template DNA. Figure 2 considers only positive PCRs and presents the probabilities of obtaining a correct genotype and an incorrect genotype (e.g. only allele A) using different quantities of template DNA. The probability of obtaining only allele A decreases rapidly when using more template DNA.

Genetic typing

Table 2 summarizes the results of the genetic typing. From 50 independent typing experiments for three concentrations of template DNA, the numbers of positive PCRs are 44, 40 and 17 for the 1:1, 1:2 and 1:4 extracts respectively. The number of correct genotypes obtained was also correlated with the concentration of template DNA: 21 correct genotypes were obtained using the 1:1 extract, 16 using the 1:2 extract and only three using the 1:4 extract.

Table 1. Stochastic distribution of alleles A and B among 10 tubes, using PCR templates equivalent to the amplifiable DNA content of 1/2, 1 and 2 cells

	DNA of 1/2 cell per PCR	DNA of 1 cell per PCR	DNA of 2 cells per PCR
Tube 1	BB	BBB	BBBB
Tube 2	A	B	AAA
Tube 3		AA	AB
Tube 4	BA	AAB	BAAA
Tube 5	BB	AAB	A
Tube 6	A	BA	BABABB
Tube 7		BA	ABBBABA
Tube 8	A	BB	ABB
Tube 9			BAAAB
Tube 10		BAB	AABBBAAA
No PCR product	4	1	0
Allele A only	3	1	2
Allele B only	2	3	1
Correct genotype	1	5	7

The stochastic distribution and the analysis of the results have been determined according to the mathematical model presented in Materials and Methods.

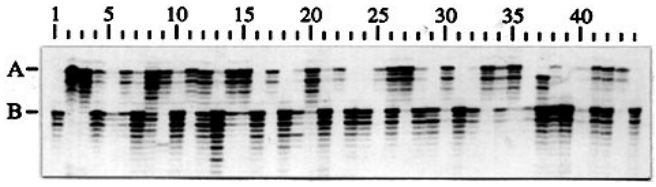

Figure 3. Autoradiograph showing the results of 50 genotyping experiments using the same extract (1:1) as template. Only the 44 positive amplifications are shown. Both alleles A and B are represented in the experiments of lanes 4–8, 10, 12–15, 19, 21, 26, 28, 30, 32, 34, 38 and 40–42. Only one allele, A or B, is represented in the experiments of lanes 1–3, 9, 11, 16–18, 20, 22–25, 27, 29, 31, 33, 35–37, 39, 43 and 44. Lanes 19 and 37 show two obvious false alleles. Lanes 3, 13, 20 and 34 may also contain false alleles.

Table 2. Results of 50 genetic typing experiments at a heterozygous locus for three different concentrations of template DNA

	Extract 1:1	Extract 1:2	Extract 1:4
No PCR product	6	11	33
Allele A only	12	11	5
Allele B only	11	12	8
Correct genotype	21	16	3
Total	50	50	50

Multiple χ^2 calculations were used with different expected results corresponding to different units of template DNA in order to test the null hypothesis that the results obtained in the laboratory experiments were not significantly different from the simulation results obtained using our mathematical model. The minimum value of χ^2 was obtained for 1.2, 0.6 and 0.3 U template DNA for the 1:1, 1:2 and 1:4 extracts respectively. The null hypothesis cannot be rejected as the 'observed' results obtained in the experiments are not significantly different from the 'expected' results of the simulation ($\chi^2 = 8.68$, df = 5). In addition, this suggests that the amount of 1:1 extract DNA used in each PCR was ~1.2 equivalents of the DNA content of one diploid cell.

Figure 3 presents the genotypes corresponding to the 44 positive PCRs from the 1:1 extract and clearly shows that a single PCR is not sufficient to obtain a reliable genotype result. Lanes which contain both alleles can exhibit a significant variation in intensity between them. Two obvious cases of false alleles are visible in lanes 13 and 19. The relative intensity of the main band and of the first shadow band below also suggests that lanes 3, 13, 20 and 34 may contain a false allele 2 bp shorter than the true allele. Using the 101 positive PCRs obtained for the three concentrations of template DNA, we estimate that a particular extra allele was not observed in >5% of the experiments.

DISCUSSION

A mathematical model has been developed to design a procedure to obtain reliable genotyping based on PCR when using very low quantities of template DNA. This model takes into account the random sampling of template molecules in the extract and assumes that a single template molecule can be detected. The results of 150 typing experiments clearly show that the genotyping of very dilute DNA samples follows a stochastic process (Fig. 3 and Table 2), consistent with the proposed model.

Random sampling of template DNA

Based on our proposed mathematical model, the probability of obtaining a correct genotype at the 99% confidence level corresponds to the use of 8 U template DNA, which is ~56 pg DNA for mammals. However, the probability of obtaining a PCR product at the 99% level is reached at only 2.4 U template DNA. Therefore, when using between 2.4 and 8 U template DNA, there is a high risk of not detecting one allele, despite obtaining positive PCRs in almost all experiments. This problem is illustrated in Figure 1 by the gap between the two curves corresponding to the probability of obtaining a positive PCR and of obtaining a correct genotype. In our experience, when working with bear hairs and feces collected in the field, the amount of template DNA used per PCR is usually <8 U and therefore the risk of missing one allele is almost always present.

The problem of false alleles

Excluding the possibility of sporadic contamination, as explained in the Introduction, an alternative explanation can be proposed for the observation of false alleles. Amplification of microsatellite loci often produces shadow bands below the main band. These artifacts correspond to smaller numbers of repeats in the microsatellite array (19–21) and could result from slippage during the amplification process. If a single target DNA molecule is used as template and if such a slippage occurred during the first cycle of the PCR, then this artifact could be amplified in approximately the same proportion as the true target molecule and would be visible on the autoradiograph. If this hypothesis is true, then the occurrence of false alleles should be proportional to the intensity of shadow bands observed in microsatellite loci and the most common false alleles should correspond to the most intensive shadow bands. In addition, all intermediates between the most intensive false alleles (due to slippage during the first cycle) and very faint false alleles (due to slippage during the following few cycles) should be observed. At present, we have carried out ~1000 typing experiments involving dinucleotide repeat loci using brown bear hair or feces as a source of DNA. Despite the difficulty in scoring false alleles due to large variations in band intensity, the occurrence and the length of the false alleles observed seem to support all three of the assumptions above (data not shown).

Guidelines when genotyping very dilute DNA samples

The guidelines we propose for genotyping very dilute DNA samples are only valid under the following conditions: (i) a single target molecule can be detected; (ii) the amount of template DNA is very low, in the picogram range, but is not accurately known. Our goal was to obtain a reliable genotyping, with a confidence level of 99%, taking into account the stochastic sampling of template DNA, the possibility of generating false alleles and the risk of contamination. One approach would be to concentrate the extract and to perform a single PCR per locus. The results of the computer simulation demonstrate the dangers of the one tube approach with 'concentrated' extract. The problem is that the quantity of extracted DNA is unknown and this quantity could be high enough to give a PCR product, but insufficient for a reliable

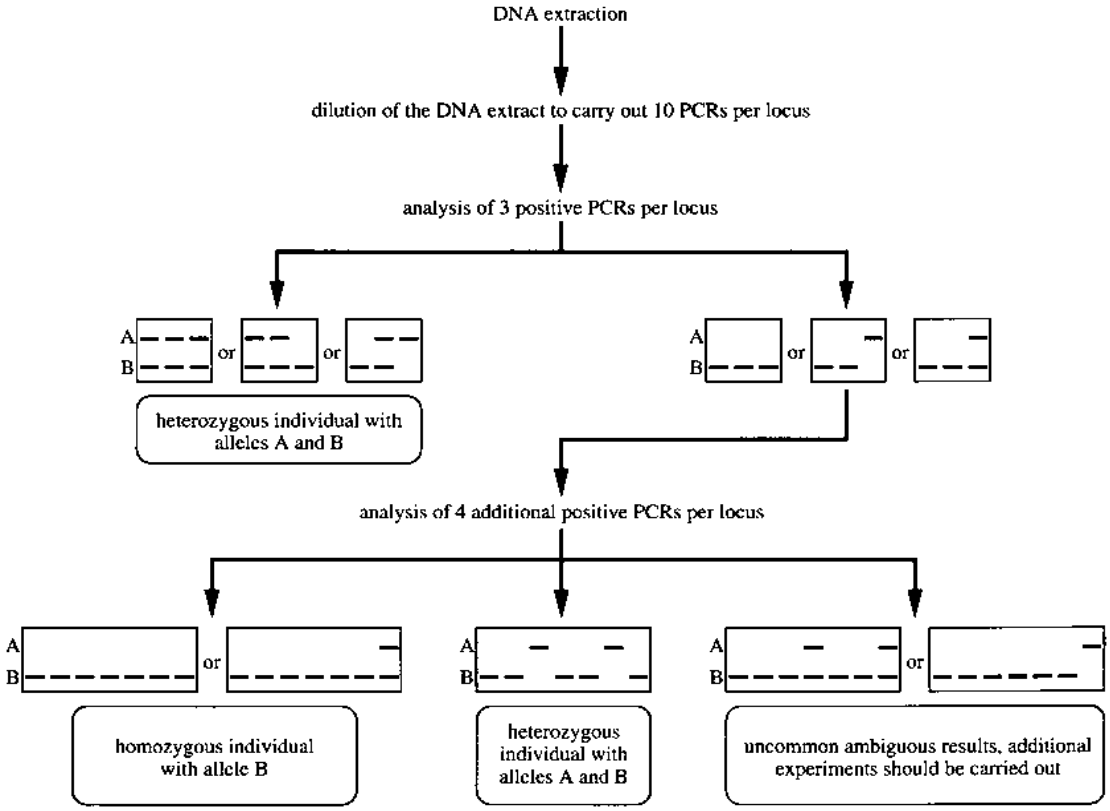

Figure 4. Flow chart diagram of the procedure used to obtain a reliable genetic typing with a confidence level of 99%.

genotyping (Fig. 1). Therefore, we propose the multiple tubes approach as a more reliable method that can monitor the three potential sources of errors: stochastic sampling, false alleles and sporadic contamination. When using the multiple tubes approach, it is important to determine the number of experiments that are necessary to obtain a reliable genotype.

The results of the simulation (Figs 1 and 2) help to design an appropriate experimental procedure. Figure 4 describes the procedure we suggest to obtain a confidence level of 99%. In this case, three positive PCR were analysed and then, depending on the results, additional experiments could be carried out. This two-step procedure was designed to avoid analysing too many positive PCRs if the amount of template DNA available is compatible with a reliable genotyping using only three experiments. In order to avoid incorrect genotypes with false alleles or sporadic contamination, our first rule was to record an allele only if it was observed at least twice. Based on our estimates, the probability of obtaining a particular extra allele (contamination or a false allele) does not exceed 5%, thus the probability of obtaining this same extra allele in two independent PCRs is <1%. To identify homozygous samples, our second rule was to score an individual as a homozygote only if seven independent experiments detected the same allele. Our choice of seven experiments was based on the simulation results presented in Figure 2: the curve of the probability of obtaining only one allele among positive PCRs has a maximum at 0.5 for very dilute template DNA. Thus, the probability of obtaining only one out of the two alleles of a heterozygous individual is 0.5^n, where n is the number of independent experiments. When $n = 7$, the probability of detecting only one of two alleles is <1%, corresponding to a confidence level of 99% for characterizing homozygous individuals. Because these calculations were based on the maximum probability of detecting only one of two alleles, $n = 7$ is a conservative estimate.

In all our experiments with bear feces and hair, it has been possible to determine the genotype of an amplifiable sample with a 99% confidence level using rules one and two as decribed above. However, after seven independent experiments, it is theoretically possible to obtain ambiguous results (Fig. 4). For example, a single allele may be observed in six experiments and a different single allele may be observed in another experiment. In such cases, we recommend that some additional experiments are performed to determine if the allele that was observed only once is a false or a true allele. Similarly, if a particular allele is present in all seven experiments and a second allele is observed in two experiments, then we recommend additional experiments, particularly if the second allele is one repeat shorter than the most common allele and could therefore be formed by a PCR slippage event.

Based on the results of the computer simulation, we estimated that at least 5 U template DNA/locus (~35 pg in mammals) are necessary to follow the procedure outlined in Figure 4 and to achieve a genetic typing with a 99% confidence level (this corresponds to 10 independent PCRs, with 0.5 U amplifiable template DNA/reaction). Our proposed mathematical model can be used to design other guidelines according to the level of confidence needed. It is also interesting to note that the proportion of positive PCRs is a poor indicator of the amount of DNA used as template when more than ~1 U is used (Fig. 1). As explained above, the fact that all or almost all PCRs work does not mean that

the genotyping is reliable. However, the fact that some PCRs do not work is a strong indication that the risk of incorrectly scoring a heterozygous individual as a homozygous individual is very high.

Based on the results obtained in this study, the multiple tubes procedure represents the best approach for obtaining reliable genotypes when using samples with very small and unknown DNA quantities. Therefore, the multiple tubes procedure should be systematically used when genotyping nuclear DNA loci of fossils, museum specimens, forensic samples, or hair and feces samples of free ranging animals.

ACKNOWLEDGEMENTS

We would like to thank Christiane Dubois-Paganon for her valuable technical help during the genetic analysis. This work was supported financially by the University Joseph Fourier, the Centre National de la Recherche Scientifique, the French Ministry of the Environment (DRAEI-EGPN and DNP) and a LIFE Programme of the European Union.

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