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SHORT COMMUNICATION

Plucked hair samples as a source of DNA: reliability of dinucleotide microsatellite genotyping

B. GOOSSENS,† L. P. WAITS and P. TABERLET
Laboratoire de Biologie des Populations d'Altitude (CNRS UMR 5553), Université Joseph Fourier, BP 53, 38041 Grenoble Cedex 9, France

Abstract

To test whether plucked hairs are a reliable source of DNA for genotyping microsatellite loci, we carried out experiments using one, three, or 10 hairs per extract for 50 alpine marmots. For each extract, seven independent genotypings were performed for the same locus (multiple-tubes approach). Two types of genotyping errors were recorded: a false homozygote defined as the detection of only one allele of a true heterozygote, and a false allele defined as a PCR-generated allele that was not one of the alleles of the true genotype. Using DNA extracted from one, three, or 10 hairs, the overall error rate was 14.00%, 4.86%, and 0.29%, respectively. Based on our results, we conclude that 10 hairs should be used to obtain consistently reliable genotypings using the single-tube approach, and that a single plucked hair could represent a reliable source of DNA if the multiple-tubes approach is used. For future studies of dinucleotide repeat diversity using DNA extracted from one to three shed or plucked hairs, we strongly recommend initiating an appropriate pilot study to quantify the error rate and to determine the reliability of the single-tube approach.

Keywords: genetic typing, hair, *Marmota marmota*, multiple-tubes approach, noninvasive genetic sampling, polymerase chain reaction

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Introduction

Noninvasive sampling of hairs collected in the field has become an important tool for molecular genetic studies of free-ranging mammals (e.g. Taberlet & Bouvet 1992; Morin & Woodruff 1996; Taberlet *et al*. 1997). Collecting plucked hairs could also be an alternative genetic sampling strategy. This is an attractive alternative as it requires less skill, time, and money than collecting blood or biopsy samples. Furthermore, for endangered species, it is much easier to obtain a sampling permit when collecting hairs. However, one drawback to using shed or plucked hairs is that the amount of DNA extracted is much lower than when using blood or tissue. Under these limiting conditions, it has been demonstrated that obtaining PCR products is much easier than obtaining reliable genotypings (Navidi *et al*. 1992; Taberlet *et al*. 1996).

To study the mating system of the alpine marmot (*Marmota marmota*, Sciuridae) and to evaluate the degree of extrapair paternity in the family groups of this social rodent, we are studying microsatellite polymorphism of DNA extracted from hairs taken directly from the animals during trapping and marking for behavioural field studies (Goossens *et al*. 1996). When we compared known mother–offspring relationships defined by field data with genotyping results, we observed single-locus genotypes where an offspring did not share an allele with the mother. Additional experiments for the same loci and the same individuals revealed the missing alleles. Therefore these inconsistencies could not be attributed to the presence of null alleles (Callen *et al*. 1993) or contamination, indicating that other types of genotyping errors may have occurred.

A number of other studies have also raised concerns about the potential for genotyping errors when amplifying nuclear DNA microsatellite loci from low DNA quantities. Under these limiting conditions, two main genotyping

Correspondence: B. Goossens. †Present address: The Zoological Society of London, Institute of Zoology, Regent's Park, London NW1 4RY, UK. Fax: + 44 171-5862879; E-mail: b.goossens @ucl.ac.uk

*Present address: Fish and Wildlife Resources University of Idaho, Moscow, ID 83844–1136, USA.

errors can occur and will lead to spurious results. First, one allele of a heterozygous individual may not be detected (Navidi *et al*. 1992; Gerloff *et al*. 1995; Taberlet *et al*. 1996, 1997; Gagneux *et al*. 1997). Second, PCR-generated alleles or 'false alleles' may arise (Foucault *et al*. 1996; Taberlet *et al*. 1996). These difficulties could potentially be overcome by repeating experiments several times for each locus and for each extract (multiple-tubes approach: Navidi *et al*. 1992; Taberlet *et al*. 1996). For homozygous individuals, a confidence level of 99% in genotyping assignments will be reached by analysing seven independent PCRs (Taberlet *et al*. 1996). However, the implementation of the multiple-tubes approach is expensive and time-consuming because many samples need to be genotyped at multiple loci.

In this paper, we focus on two important questions concerning the utility of plucked hairs as alternative genetic sampling. (i) Is the single-tube approach reliable for genotyping of dinucleotide microsatellite loci using plucked hairs as a source of DNA? (ii) How many hair roots should be included in an extraction to obtain an error rate of 1% or less using the single-tube approach and the multiple-tubes approach?

Materials and methods

We estimated the error rate when using plucked hairs as a source of DNA by performing a large number of genotyping experiments, and comparing the results obtained with the true genotypes. In order to test the influence of increasing DNA quantities on the genotyping reliability, we analysed 50 randomly chosen marmot individuals, using one, three, and 10 hair roots in each DNA extract. Seven genotyping experiments were performed for the same microsatellite locus for each DNA extract (multiple-tubes approach). Using such a procedure, a total of 21 typing experiments were performed for the same locus and for each individual, and this allowed us to deduce the true genotypes with a very high confidence level ($P < 0.000001$ according to Taberlet *et al*. 1996).

The sampling was conducted in three different locations of the French Alps (National Park of La Vanoise, National Park of Les Ecrins, Maurienne Valley). Since 1990, 588 marmots have been captured using live traps, and 50–100 hairs were plucked from each individual and stored in paper envelopes at room temperature until DNA extraction. Out of these samples, 50 individuals were randomly chosen for this study.

DNA was extracted from one, three and 10 hairs per individual by cutting a 1-cm portion from the root end of the hair and placing it in 400 µL of a 5% Chelex-100 (Bio-Rad) suspension (Walsh *et al*. 1991). The samples were incubated at 56 °C for 5–6 h, and then in a boiling-water bath for 8 min. Five µL of each extract was added as template in each PCR reaction.

The multiple-tubes procedure was conducted using the primers for locus SS-Bibl1 (primer sequences designed from Klinkicht 1993; Goossens *et al*. 1996). Amplification of this locus for each individual was carried out in a 25 µL reaction (10 mM Tris-HCl (pH 9.0), 200 mM $(NH_4)2SO_4$, 50 µM each dNTP, 1.5 mM $MgCl_2$, 5 ng of BSA, 0.1 U Amplitaq® Gold DNA polymerase (Perkin Elmer), 0.5 µM fluorescent primer SS-Bibl1Forward (5'-6FAM*CTGAAG-CAGCCATCCAGTA-3'), 0.5 µM nonfluorescent primer SS-Bibl1 Reverse (5'-ATGGTTCTCCACCACTAGC-3'), 5 µL extract). A PCR amplification of 50 cycles was carried out (initial denaturation 95 °C for 10 min, 95 °C for 15 s, 55 °C for 15 s, 72 °C for 30 s) using Perkin Elmer Gene Amp PCR System 9600. In these conditions, a single template molecule can be detected (Rameckers *et al*. 1997). PCR products were visualized on an agarose gel (SeaKem) to detect positive PCRs and to estimate the dilution factor for the subsequent step. All positive PCR products were separated in a 6% Long RangerTM gel (FMC) at 2670 V for 2.5 h using an ABI PRISM™ 377 DNA sequencer (Perkin Elmer) with marker GS350 Tamra. All gels were analysed using GeneScanTM Analysis 2.0. and GenotyperTM 1.1. software.

We estimated the DNA quantity of 20 10-hair chelex extracts. Fifty µL of the chelex supernatant was mixed with 50 µL of Pico Green® solution (Molecular Probes), incubated at room temperature for 10 min, and the fluorescence was measured on a multiwell plate reader (Perseptive Biosystems) at a sensitivity of seven. The standard curve was obtained by using a known DNA quantity in a chelex supernatant without hair.

Results

The genotypes at locus SS-Bibl1 were unambiguously identified by the analysis of 21 independent PCRs per individual (according to Taberlet *et al*. 1996). Thirty-five heterozygous individuals and 15 homozygous individuals were detected, and observed heterozygosity of this random sample was 70%. Two types of errors were detected: false homozygotes and false alleles (Fig. 1). Table 1 summarizes the errors observed. When using only one hair for the DNA extraction, 35 false homozygotes, 14 PCRs with false alleles, and one negative PCR were observed out of the 350 PCRs. When using three hairs, 12 false homozygotes and five PCRs with false alleles were detected. When using 10 hairs, only one false homozygote was detected in 350 PCRs. In this case the unrecorded allele was present, but the intensity of the allele was about 20-times weaker than the recorded allele. The total genotyping error rate for this microsatellite locus was therefore 14.00% (95% confidence interval: 10.36–17.64%) for one hair; 4.86% (2.60–7.11%) for three hairs; and 0.29% for 10 hairs.

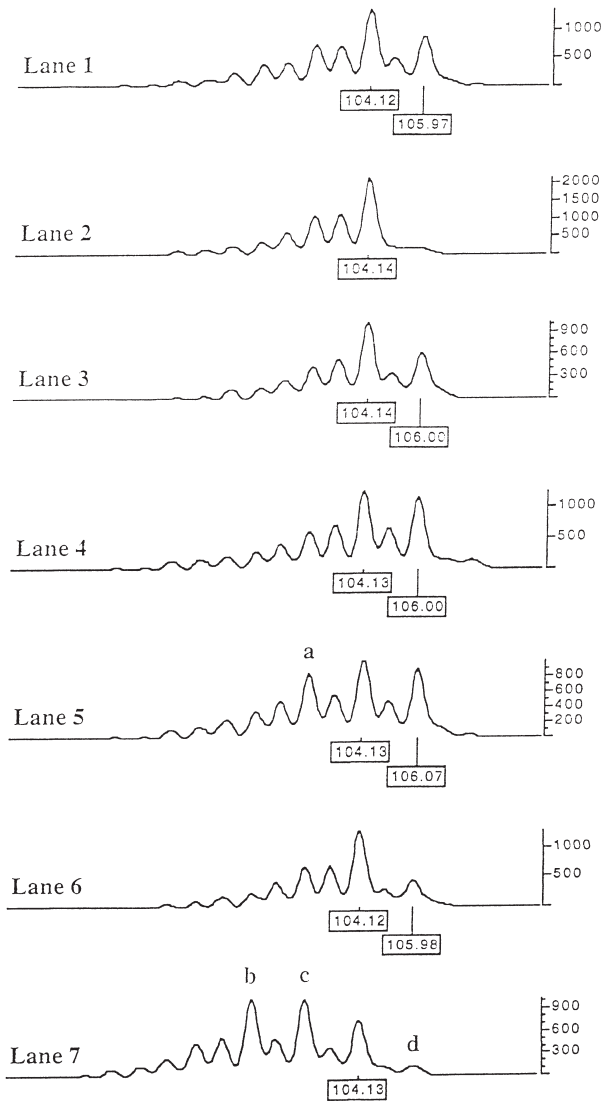

Fig. 1 Electropherograms showing the two types of errors that were observed when using a single marmot hair as a source of DNA. Lanes 1–7 correspond to seven independent DNA amplifications using the same extract as template. The true genotype of this heterozygous individual is composed of alleles 104 and 106. Lane 2 shows a false homozygote where allele 106 was not amplified. Lane 5 shows an ambiguous peak (a) which could correspond to a false allele. Lane 7 shows two obvious false alleles (peaks b and c), and the allele 106 (peak d) is too weak to be reliably recorded. The horizontal scale represents the number of base pairs and the vertical scale represents the number of fluorescent units.

Figure 2 illustrates the relative variation in peak intensities between two alleles of a heterozygous individual relative to the number of hair roots used for the extraction. Clearly, the variation in peak intensities is much higher when using fewer hair roots in the DNA extraction. The DNA quantifications indicate that each plucked marmot hair provides 0.91 ng (± 0.44 ng) of DNA.

Discussion

This study demonstrates that when using the single-tube approach with a DNA extract from a single plucked hair, genotyping errors at dinucleotide microsatellite loci are substantial and can lead to inaccurate results. In these conditions, our results suggest that the amount of template DNA used in each PCR (1/80 of the total extract) is in the picogramme range, and this low quantity of DNA is at the origin of the 14.00% genotyping errors. By using three or 10 hairs, the error rates can be lowered to 4.86% or 0.29%, respectively. It is also possible that altering the protocol to concentrate the DNA may decrease the error rate. Thus, when performing a similar protocol to extract DNA from one to three hairs the confidence level obtained using the single-tube approach is incompatible with reliable individual identification, paternity assessment, and estimation of heterozygosity. However, our results also show that when using the multiple-tubes approach (with seven PCRs) all 50 individuals were genotyped correctly, even in the case of the one-hair extract.

As expected according to previous studies (Foucault *et al*. 1996; Taberlet *et al*. 1996), the number of false homozygotes and false alleles increases when less template DNA is used. This statement is based on the assumption that extracts involving one, three, and 10 hairs contain increasing DNA amounts. This assumption is confirmed by the error rates observed for the 1050 experiments. However, the observation that for at least one individual the error rate was much higher for three hairs (five false homozygotes out of seven) than for one hair (no error) suggests that the amount of DNA can vary, perhaps by one order of magnitude, among different hairs. This hypothesis is also supported by the high variance observed in the DNA quantifications of 20 10-hair extracts.

The expected DNA quantity that can be extracted from one plucked marmot hair (0.91 ng) is consistent with the

	Number/(%) of error		
Error type	1 hair	3 hairs	10 hairs
PCRs with false alleles (350 PCRs)	14/(4.00%)	5/(1.43%)	0/(0.00%)
PCRs with false homozygotes (245 PCRs)	35/(14.29%)	12/(4.90%)	1/(0.41%)
Total genotyping errors (350 PCRs)	49/(14.00%)	17/(4.86%)	1/(0.29%)

Table 1 Genotyping error rates obtained when using one, three, or 10 plucked marmot hairs as a source of DNA for 50 randomly chosen individuals. Seven PCRs were performed for each DNA extract, and this random sample includes 35 heterozygotes and 15 homozygotes

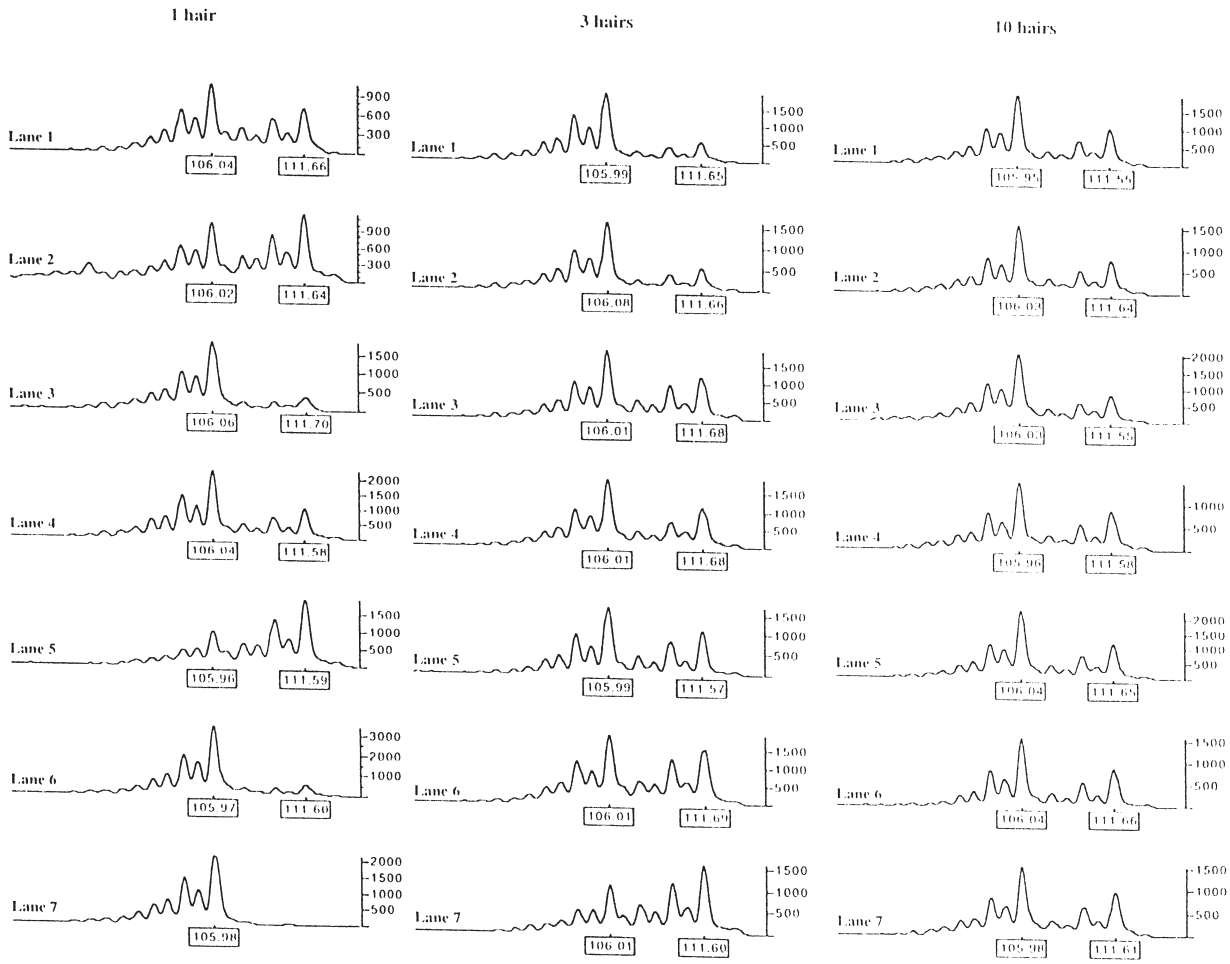

Fig. 2 Electropherograms showing the variation in peak intensity between two alleles according to the number of hairs used for the DNA extraction. Lanes 1–7 correspond to seven independent DNA amplifications using the same extract as template. The true genotype of this heterozygous individual is composed of alleles 106 and 112. Clearly, when the number of hairs in the DNA extract increases, the relative peak intensity between the two alleles becomes more homogenous. Lane 7 for one hair shows a false homozygote where allele 112 was not amplified. The horizontal scale represents the number of base pairs and the vertical scale represents the number of fluorescent units.

estimation of Gagneux *et al*. (1997) for shed hairs of chimpanzees. The observed error rate for false homozygotes that are expected with such a quantity (11.4 pg per PCR for the one-hair experiments) should be about 30% when using the model of Taberlet *et al*. (1996). Our error rate in such conditions is only 14.29%, which means that the DNA quantity per marmot hair could be largely underestimated, probably in relation to the difficulty of DNA quantification in limiting conditions.

The false homozygotes obtained, due to stochastic sampling of alleles in the very diluted extract, can occur when PCR conditions allow the detection of a single target molecule. We believe that the potential for observing false homozygotes will increase if the efficiency of the amplification is not the same for each allele, as could be the case when one allele is much longer than the other. This problem could explain why we observed one false homozygote with the 10-hair extract as we missed an allele which was consistently less intense in the other experiments.

The false alleles (PCR-generated alleles) probably correspond to slippages (Schlötterer & Tautz 1992; Foucault *et al*. 1996) during the first few cycles of the amplification (Taberlet *et al*. 1996) and are detected at the same intensity as true alleles only when the number of target molecules is very low. This is consistent with the high variation in band intensities when the amount of template DNA decreases (Fig. 2), and could explain why we did not observe false alleles with the 10-hair extracts. This type of error, which cannot be distinguished from sporadic contaminations, could possibly be lowered by analysing tri- or tetranucleotide microsatellites which are known to be less prone to slippage artefacts than dinucleotide repeats (see Edwards *et al*. 1991).

This study was conducted on the alpine marmot and our results may be species specific as the amount of DNA present on the root of one hair could be variable among different mammalian species. Thus, it is difficult to directly use our results for designing genotyping strategies in another species. Furthermore, our results only concern plucked hairs, and it is very likely that shed hairs collected in the field would produce a higher level of errors, making the genotyping of free-ranging mammals difficult, as recently observed for the brown bear (*Ursus arctos*) in the Pyrenees (Taberlet *et al*. 1997).

To summarize, typing experiments using plucked marmot hairs as a source of DNA show that when the amount of template DNA is very low, the reliability of genotyping can be compromised due to: (i) the possibility of missing one allele of a heterozygous individual; (ii) generating PCR artefacts which can be misinterpreted as true alleles; and (iii) the high variation in peak (or band) intensity between the two alleles of the same locus. Despite these difficulties, a single plucked hair could represent a reliable source of DNA if the multiple-tubes approach is used. When using the single-tube approach, we strongly recommand carrying out an appropriate pilot study to quantify the level of genotyping errors in order to determine if the error rate is low enough to accurately assess familial relationships, identify individuals, and/or estimate levels of genetic diversity.

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