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BRIEF COMMUNICATION

Evaluation of methods for single hair DNA amplification

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Abstract Because of the low amount of DNA in single hairs, it may be difficult to obtain reliable genotypes for forensic and conservation genetics studies. We therefore compared different methods for reliably genotyping single hair samples. Our results indicate that preliminary whole genome amplification can increase the likelihood of successfully genotyping a single hair compared to other commonly used protocols. The difference between the methods is small for single locus comparisons, but it becomes more important in multi-locus comparisons. The economic and time costs of the whole genome amplification may prevent its large-scale use in non-invasive monitoring programs. Nevertheless, it may be a very useful approach for the analysis of especially valuable samples.

Keywords Noninvasive monitoring · Whole genome amplification · Chelex · GenomiPhi · Dropout

Introduction

Hairs are often used as a source of DNA for genetic typing in studies of free-ranging mammals and in forensic research (Taberlet and Bouvet 1992; Taberlet et al. 1997; Vigilant 1999). However, the drawback of using hair instead of using e.g. blood or biopsy samples is the risk of genotyping errors due to the very low amount of DNA available (Taberlet et al. 1996, 1999). The most common errors are allelic dropout (one allele in a heterozygote locus fails to amplify) or the appearance of an extra allele (false allele) due to sporadic contamination or slippage during the first cycles of amplification (PCR-generated allele) (Taberlet et al. 1996, 1999). To minimise these problems, cutting the hair shafts as close to the root as possible can help to reduce the amount of PCR inhibitors in the DNA extract, which otherwise could induce allelic dropouts (Gagneux et al. 1997; Taberlet et al. 1999). Using tri- and tetranucleotide microsatellites instead of dinucleotide microsatellites may reduce the risk of false alleles (lower risk of slippage) (Goossens et al. 1998; Taberlet et al. 1999). The multiple-tubes approach (each sample is genotyped several times for each locus) can also reduce the frequency of genotyping errors (Goossens et al. 1998; Taberlet and Waits 1998; Taberlet et al. 1999).

During the last few years several methods for whole genome amplification have been developed. These methods are intended to amplify the entire genome starting from a limited amount of good quality DNA, as could be obtained, for example, from single hairs. Although this could potentially facilitate forensic and conservation research, it is not known to what degree genotyping errors ought to be expected. In this study we compared the rate of microsatellite genotyping errors incurred while genotyping single hairs using conventional protocols with that following whole genome amplification. We assessed what method was most likely to provide the correct genotype using only one single hair root.

Material and methods

The methods tested were a standard hair DNA extraction using Chelex® 100Resin (BioRad Laboratories,

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Hercules, CA, USA; *extraction alone*) (see Walsh et al. 1991), a whole genome amplification using GenomiPhi DNA Amplification Kit (Amersham Biosciences, NJ, USA) applied to the hair root (*WGA alone*) and a standard Chelex extraction followed by a whole genome amplification of the extract using GenomiPhi DNA Amplification Kit (e*xtraction + WGA*). The extracted/amplified DNA was then typed for nuclear microsatellite markers using identical protocols.

The Chelex extraction method is frequently used to release DNA from a low numbers of cells by a boiling treatment, and at the same time protecting the DNA from the boiling effects with resin beads (Walsh et al. 1991). The GenomiPhi DNA Amplification Kit can be used either to amplify DNA directly from a specific material (unpurified cell lysates), or to re-amplify purified DNA from an earlier extraction.

The comparison of methods was based on freshly plucked large guard hairs from the back and tail of two dogs. Hairs were processed immediately after plucking. One single hair with a visible follicle was used for each test. DNA was extracted from 16 hairs (8 from each dog) using Chelex and directly amplified from 16 more hairs using GenomiPhi. One microliter of the DNA obtained from the Chelex extractions was also used in whole genome amplification with GenomiPhi. An additional tube containing no hair (negative control) was added to each extraction/amplification to detect possible contamination. This tube was treated in the same way as the hair samples. No contamination was detected in any case.

For the Chelex extraction, the hair was put in 200 µl hair buffer (0.9 g Polyoxyethylene 10 Lauryl Eter, 5 g Chelex® 100Resin 100–200 mesh sodium form analytical grade, 1 ml 1 M Tris–HCl pH 8.0 and water up to 100 ml) and was digested with 20 µg of proteinase K and 7 µmol DTT. The sample was vortexed and incubated for 3–6 h at 56°C (until it was completely digested). Then the mix was vortexed again and incubated at 100°C for 8 min to inactivate enzymes. The sample had a final vortex followed by one minute in the centrifuge at 10,000 rpm to separate the resin beads from the DNA extract (Suenaga and Nakamura 2005).

For the GenomiPhi amplification we followed the manufacturer's protocol. One microliter of the DNA lysate (for *WGA alone*) or 1 µl from the Chelex extraction (for *extraction + WGA*) was used in the whole genome amplification step. Products of whole genome amplifications were diluted 1:100 before microsatellite amplification to obtain a concentration comparable to that with the Chelex extraction. Some tests indicated that the genotyping success rate was highest with this dilution (data not shown).

Eight biparentally inherited autosomal microsatellites, distributed across the canine genome, were typed for each sample and method: c2017 (Francisco et al. 1996), u109, u173, u225 and u250 (Ostrander et al. 1993) and PEZ05, PEZ06 and PEZ12 (Perkin-Elmer, Zoogen; see NHGRI Dog Genome Project at http://www.research.nhgri.nih.gov/dog_genome/). The PCR amplifications for the microsatellites were done in five 10 µl reactions: two multiplexes ({u109, u173, u225} and {PEZ06, PEZ12}) and the remaining loci separately. The PCR mix included 1xHotStar buffer (QIAGEN, Hilden, Germany) containing 1.5 mM $MgCl_2$, 0.25 mM dNTP, 0.32 µM of each primer, 2.0 mM $MgCl_2$, 0.05 µl 5 × Q solution, 0.45U HotStarTaq and 2 µl DNA template. The PCR profile included an initial denaturation step at 95°C for 15 min followed by 12 touchdown-cycles (30 s of denaturation at 95°C, 30 s annealing starting at 58°C and decreasing 0.5°C each cycle, followed by extension at 72°C for 1 min), 38 cycles of amplification (denaturation at 95°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 1 min) and an additional extension step at 72°C for 15 min. Three PCR replicates were performed for each microsatellite locus.

The PCR products were pooled in two different pools (pool A: c2017, u109, u173 and u225; pool B: u250, PEZ05, PEZ06 and PEZ12) and run on the MegaBACE1000™ (Amersham Biosciences) (Fig. 1). The genotypes were analysed using Genetic Profiler v2.2 (Amersham Biosciences).

To identify an individual's genotype after three replicates we followed the same criteria as Hedmark et al. (2004): for a homozygote all three replicates had to show the same homozygote genotype; for a heterozygote, at least two of the three replicates should show the same heterozygous result. Failure to fulfil these conditions led to failed genotype identification. Since all hairs came from the same two dogs, we could easily determine if the obtained genotype was correct or not.

Statistic comparisons were made in VassarStats: Web Site for Statistical Computation (http://faculty.vassar.edu/lowry/VassarStats.html). Yates χ^2 test was used, which is corrected for continuity. These tests were only performed if all expected frequencies were equal to or greater than 5.

Result and discussion

A total of 1152 microsatellite amplifications were analysed for this study (Table 1). Eight hairs and three replicate PCRs for each one of three methods

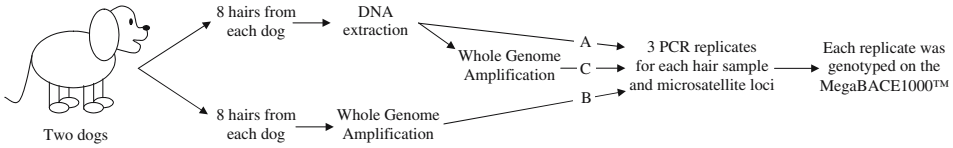

Fig. 1 Flow diagram representing the experimental procedure. The methods compared are *extraction alone* (**A**), *WGA alone* (**B**) and *extraction + WGA* (**C**)

gave 72 replicates per marker and individual, which allowed unambiguous genotyping of both individual dogs at all loci. Of the eight microsatellites used, four loci were homozygote and four were heterozygote for one dog (purebred) and three were homozygote and five were heterozygote for the other dog (mixbred).

We calculated the proportion of failed PCR amplifications (without any PCR product) for each method. *Extraction alone* resulted in more failed amplifications than the other methods, the difference being significant with *WGA alone* ($P = 0.03$, Table 1).

The number of successful (providing the correct genotype) homozygote replicates, was highest for *WGA alone* although the difference between the methods was not significant ($P > 0.05$). Counting the number of successful heterozygote replicates instead showed that *extraction + WGA* had the lowest success rate compared to *WGA alone* ($P = 0.001$, Table 1) and *extraction alone* ($P = 0.02$, Table 1).

In the heterozygote replicates we also counted the number of dropouts. *Extraction + WGA* is more prone to dropouts compared to the other two methods, 29.6% dropouts were found. This was significantly higher than both *extraction alone* (7.4%, $P < 0.001$) and *WGA alone* (5.1%, $P < 0.001$). *WGA alone* and *extraction alone* showed much fewer dropouts compared to two

Table 1 Genotyping success for single hair samples of dogs, following three different protocols

	No. of tests per method	WGA alone	Extraction alone	Extraction + WGA		WGA alone vs. extraction alone	WGA alone vs. extraction + WGA	Extraction alone vs. extraction + WGA
# Failed amplifications[a]	384	96 (25.0%)	134 (34.9%)	112 (29.2%)	χ^2 (Yates)	4.51	0.82	1.32
					P	0.034*	0.365	0.251
# Successful homozygote replicates[b]	168	126 (75.0%)	104 (61.9%)	120 (71.4%)	χ^2 (Yates)	1.07	0.04	0.55
					P	0.301	0.841	0.458
# Successful heterozygote replicates[c]	216	151 (69.9%)	130 (60.2%)	88 (40.7%)	χ^2 (Yates)	0.81	10.26	5.02
					P	0.368	0.001**	0.025*
# Allelic dropouts[d]	216	11 (5.09%)	16 (7.41%)	64 (29.6%)	χ^2 (Yates)	0.54	30.85	23.32
					P	0.462	<0.001***	<0.001***
# Successful homozygote genotypes[e]	56	40 (71.4%)	29 (51.8%)	38 (67.9%)	χ^2 (Yates)	0.79	0	0.51
					P	0.374	1.000	0.475
# Successful heterozygote genotypes[f]	72	52 (72.2%)	46 (63.9%)	30 (41.7%)	χ^2 (Yates)	0.11	3.27	1.81
					P	0.740	0.071	0.179
# Correct genotype[g]	128	92 (71.9%)	75 (58.6%)	68 (53.1%)	χ^2 (Yates)	0.86	1.93	0.13
					P	0.354	0.165	0.718

[a] Failed amplifications = no alleles were obtained. Number of tests: 384 for each method = 2 dogs*8 hairs*8 microsatellites*3 replicates

[b] Successful homozygote replicate = positive PCR replicate providing the correct genotype. No. of tests: 168 for each method = 8 hairs*3 replicates*(4 + 3) homozygote loci

[c] Successful heterozygote replicate = positive PCR replicate providing the correct genotype. No. of tests: 216 for each method = 8 hairs*3 replicates*(4 + 5) heterozygote loci

[d] Allelic dropout = one allele in a heterozygote loci is not obtained. No. of tests: 216 = 8 hairs*3 replicates*(4 + 5) heterozygote loci

[e] Successful homozygote genotype = 3 positive PCR replicates providing the correct genotype. Number of tests: 56 for each method = 8 hairs*(4 + 3) homozygote loci

[f] Successful heterozygote genotype = 2 or 3 positive PCR replicates providing the correct genotype. Number of tests: 72 for each method = 8 hairs*(4 + 5) heterozygote loci

[g] Correct genotype (in total) = sum of # successful homozygote genotypes and # successful heterozygote genotypes = 56 + 72 = 128 (number of tests)

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

other studies (using Chelex), which show 31% droupouts when using shed hair (Gagneux et al. 1997) and 14% droupouts using plucked hair (Goossens et al. 1998). These differences are likely to have resulted from the quality of the hair samples and from the lab procedures used.

Counting successfully typed homozygote genotypes (all three replicates had to give the same result), showed no significant difference between the methods ($P > 0.05$ for all comparisons). In terms of identification of heterozygote genotypes (with at least two of the replicates showing the same result), *WGA alone* had the highest success rate (72.2%) and *extraction + WGA* had the lowest (41.7%, $P = 0.07$).

We finally estimated the total number of successful genotypes after the three replicates. For *WGA alone*, 71.9% of the microsatellites were successfully genotyped, 58.6% for *extraction alone* and 53.1% for *extraction + WGA*. Although *WGA alone* provided the correct genotype in a larger number of cases, the difference was not significant. The protocol applied to identify genotypes, based on three replicate amplifications of each microsatellite locus (Hedmark et al. 2004), never led to the identification of a wrong genotype with any of the methods.

Our results show that it is in many cases possible to reliably genotype single hair samples. PCR amplification was successful in 65–75% of the cases depending on the method used. The probability of obtaining the correct consensus genotype following the criteria in Hedmark et al. (2004) varied between 53 and 72%, similar to that in three other studies with single hairs (shed or plucked), where the success rate of obtaining the correct genotype varied between 33 and 86% (Gagneux et al. 1997; Goossens et al. 1998; Vigilant 1999). Although a low concentration of template DNA can lead to a high proportion of unsuccessful amplification and an accumulation of allelic dropouts (Vigilant 1999), our results show that genotyping is still possible in many cases with the limited amount of DNA provided by one single hair.

The results indicate that *WGA alone* performs consistently better through all the tests than the other methods (both looking at individual loci and to the sum of all of them), although the only significant difference between this method and *extraction alone* was the number of failed PCR amplifications. However, these comparisons reflect the success rate in locus-by-locus comparisons. Comparing the multilocus genotypes obtained for each hair, the likelihood of obtaining complete genotypes is higher for *WGA alone*. Out of 16 hairs genotyped (8 from each dog) correct genotypes for 7 or 8 loci were obtained for 11 hairs (68.8%) using *WGA alone*, 8 (50%) with *extraction alone* and only 4 (25%) with *extraction + WGA*. Theoretically, if a sample of one hair was to be genotyped at 10 microsatellite loci, the probability of obtaining the correct genotype after three replicates (following the protocol of Hedmark et al. 2004) at eight loci at least is, according to a binomial distribution, 14.6% for *extraction alone* and 43.5% for *WGA alone*. This implies that for highly valuable samples whole genome amplification can notably increase (about three times) the chances of obtaining a usable genotype.

Despite this, other considerations should be taken into account when deciding what method to use. Chelex extraction is a fairly simple and cheap method (Suenaga and Nakamura 2005) while GenomiPhi DNA Amplification Kit is more expensive, has a few more steps and requires a longer incubation time for the version tested (although this does not imply handling and can be done overnight). On the other hand, the whole genome amplification produces a very high DNA concentration that can be diluted and used for a larger number of assays (see Table 2). Other versions of same whole genome amplification kit are now available with shorter incubation time and higher yield. However, these were not tested in this study.

In summary, if only a limited amount of material is available (e.g. in forensic work) or if the samples are especially valuable, whole genome amplification (*WGA alone*) previous to the genotyping work may increase the chances of obtaining a usable multilocus genotype. Also, this will provide a larger amount of DNA, which can allow further studies. However, the high cost of this method and the time it takes precludes its use in most large-scale genotyping projects.

Table 2 Comparison between the methods concerning costs, time, genotyping result and amount of extract/amplified DNA

	Costs	Time	Quality	Amount of DNA (final volume)	No. of possible PCR reactions (2 µl/reaction)
WGA alone	3.83 EUR	16.5–18.5 h	+	20 µl*dilution 1:100 = 2,000 µl	1,000
Extraction alone	0.05 EUR	3.5–6.5 h	+	Ready to use, not diluted = 200 µl	100
Extraction + WGA	3.88 EUR	20–25 h	–	20 µl*dilution 1:100 = 2,000 µl	1,000

Extraction alone is, in most cases, preferable due to its low cost and simplicity.

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References

Francisco LV, Langston AA, Mellersh CS, Neal CL, Ostrander EA (1996) A class of highly polymorphic tetranucleotide repeats for canine genetic mapping. Mamm Genome 7:359–362

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

Goossens B, Waits LP, Taberlet P (1998) Plucked hair samples as a source of DNA: reliability of dinucleotide microsatellite genotyping. Mol Ecol 7:1237–1241

Hedmark E, Flagstad O, Segerstrom P, Persson J, Landa A, Ellegren H (2004) DNA-based individual and sex identification from wolverine (Gulo gulo) faeces and urine. Conserv Genetics 5:405–410

Ostrander EA, Sprague GF, Rine J (1993) Identification and characterization of dinucleotide repeat (Ca)N markers for genetic-mapping in dog. Genomics 16:207–213

Suenaga E, Nakamura H (2005) Evaluation of three methods for effective extraction of DNA from human hair. J Chromatogr B Analyt Technol Biomed Life Sci 820:137–141

Taberlet P, Bouvet J (1992) Bear conservation genetics. Nature 358:197

Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, Waits LP, Bouvet J (1996) Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic Acids Res 24:3189–3194

Taberlet P, Camarra JJ, Griffin S, Uhres E, Hanotte O, Waits LP, Dubois-Paganon C, Burke T, Bouvet J (1997) Noninvasive genetic tracking of the endangered Pyrenean brown bear population. Mol Ecol 6:869–876

Taberlet P, Waits LP (1998) Non-invasive genetic sampling. Trends Ecol Evol 13:26–27

Taberlet P, Waits LP, Luikart G (1999) Noninvasive genetic sampling: look before you leap. Trends Ecol Evol 14:323–327

Walsh PS, Metzger DA, Higuchi R (1991) Chelex-100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10:506–513

Vigilant L (1999) An evaluation of techniques for the extraction and amplification of DNA from naturally shed hairs. Biol Chem 380:1329–1331