

TECHNICAL ARTICLE

A test of the efficacy of whole-genome amplification on DNA obtained from low-yield samples

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Abstract

Conservation and population genetic studies are sometimes hampered by insufficient quantities of high quality DNA. One potential way to overcome this problem is through the use of whole genome amplification (WGA) kits. We performed rolling circle WGA on DNA obtained from matched hair and tissue samples of North American red squirrels (*Tamiasciurus hudsonicus*). Following polymerase chain reaction (PCR) at four microsatellite loci, we compared genotyping success for DNA from different source tissues, both pre- and post-WGA. Genotypes obtained with tissue were robust, whether or not DNA had been subjected to WGA. DNA extracted from hair produced results that were largely concordant with matched tissue samples, although amplification success was reduced and some allelic dropout was observed. WGA of hair samples resulted in a low genotyping success rate and an unacceptably high rate of allelic dropout and genotyping error. The problem was not rectified by conducting PCR of WGA hair samples in triplicate. Therefore, we conclude that WGA is only an effective method of enhancing template DNA quantity when the initial sample is from high-yield material.

Keywords: allelic dropout, conservation genetics, DNA yield, GenomiPhi, whole genome amplification

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Introduction

Researchers in conservation and population genetics, especially those working on nonmodel organisms, frequently encounter problems and limitations due to DNA samples that are low in quality, quantity and/or template yield. Low-yield, low-quality DNA samples (e.g. from hairs, feathers, horn, faecal matter) are vulnerable to an array of genotyping errors such as allelic dropout and the amplification of false alleles (Foucault *et al.* 1996; Taberlet *et al.* 1996; Goossens *et al.* 1998; Bonin *et al.* 2004). Such errors can lead to incorrect parentage assignment (Hoffman & Amos 2005; Pompanon *et al.* 2005), poor estimates of population size (Creel *et al.* 2003) and inaccurate estimates of genetic variation and population structure. To a certain extent such problems can be contained by employing a

multiple-tubes approach (Taberlet *et al.* 1996) although this requires substantial amounts of template DNA, which may not be available.

The concentration and template quantity of DNA is also a limiting factor when samples need to be typed at a large number of loci, for example in gene mapping or population genomics studies, or when attempting to infer parentage in bottlenecked/genetically depauperate populations. The problems associated with low DNA quantity are particularly acute when typing single nucleotide polymorphisms (SNP) as, compared to microsatellites, more loci must be typed to obtain equivalent resolution in order to, for example determine paternity and reconstruct pedigrees (Anderson & Garza 2006). Furthermore, as long-term projects develop, research foci may change, resulting in the use of extra or different genetic markers for which more DNA will be needed.

Whole genome amplification (WGA) has the potential to provide large quantities of genomic DNA from small starting volumes (Bergen *et al.* 2005). If effective and accurate, this method of DNA amplification could prove invaluable

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for conservation and population geneticists. Previous WGA methods such as primer extension pre-amplification (PEP) and degenerate-oligonucleotide-primed (DOP) polymerase chain reaction (PCR) have been criticized for their tendency for amplification bias resulting in the dropout of alleles and genotyping errors (Lasken & Egholm 2003). PEP has successfully been used to increase the quantity of DNA extracted from single sperm where, because of sperm being haploid, amplification bias is not an issue (Zhang *et al.* 1992). This study focuses on a method of rolling-circle amplification provided by GenomiPhi (GE Healthcare). The method uses random hexamer primers and a DNA polymerase from the bacteriophage Phi29 to amplify genomic DNA. It is claimed that GenomiPhi can produce as much as 4–7 µg from just 10 ng of human genomic DNA. However, GenomiPhi is designed for use with high-quality template DNA and it would be valuable to trial WGA on poor quality samples such as those sometimes used in conservation and population genetic studies. Given that GenomiPhi requires only 10 ng template DNA, we did not specifically test the case of low-yield, high-quality DNA. We tested the reliability of the method of WGA on DNA extracted from high-quality, high-yield tissue and poor-quality, degraded, low-yield hair from a population of the North American red squirrel, *Tamiasciurus hudsonicus*, from the Kluane region in the Yukon Territory, Canada.

Methods and materials

Population, behavioural and ecological studies of the Kluane red squirrel population have been ongoing since 1986, and hair has been collected from individual squirrels since 1995. Tissue collection replaced hair collection in 2003. Because squirrels are long-lived (up to 9 years), both tissue and hair were collected for a small number of squirrels. The amount of hair available from each squirrel was variable, ranging from a single hair to > 20 hairs. All hair samples were kept at room temperature [as was standard practise at the time (Goossens *et al.* 1998)], for up to 10 years prior to DNA extraction. Hence, the DNA from these hairs is likely to be degraded (Taberlet *et al.* 1999; Roon *et al.* 2003), and some samples are likely to have a low DNA yield, because only the follicle yields significant quantities of nuclear DNA (Di Martino *et al.* 2004; McNeven *et al.* 2005). In order to perform paternity analysis to reconstruct the pedigree of the Kluane squirrel population, all hair samples need to be genotyped, if possible.

Matched hair and tissue samples were available for 48 squirrels. Hair samples were obtained from either a plucked whisker, or from hair plucked from the scruff of the neck, and stored in paper or polythene bags. Tissue samples were obtained from ear clips and stored in 1.5-mL Eppendorf tubes in 95% ethanol at 4 °C. Genomic DNA tissue extractions were performed using a standard ammonium acetate-ethanol

process (Bruford *et al.* 1998). Hair extractions were performed using the paramagnetic bead DNA-IQ System with Tissue and Hair Extraction Kit (Promega) using one to 10 hairs per sample according to the manufacturer's instructions, but with an overnight incubation. The extraction protocol took place in a dedicated clean room on a Biomek 2000 liquid handling robot (Beckman-Coulter). Eluted DNA was quantified on a FLUOstar Optima Fluorometer (BMG Labtechnologies) and normalized to a standard concentration of 10 ng/µL as suggested by the GenomiPhi protocol. Normalized DNA was stored at –20 °C. Each sample was genotyped at four microsatellite loci (Thu08, Thu14, Thu42 and Thu49). A pigtail modification was added to the reverse primer of Thu14. PCR conditions for tissue samples were performed as described in (Gunn *et al.* 2005), except that 1 mM MgCl₂ was used for Thu08. PCR conditions were modified for hairs by increasing the number of cycles to 36. Microsatellite allele length was analysed using an Applied Biosystems 3730 DNA sequencer and scored using GENEMAPPER 3.7 software. The average concentration of DNA obtained was 60 ng/µL from tissue samples and 5 ng/µL from hair samples.

One microlitre of the DNA extracted from each of 48 hair samples and from 19 randomly chosen, matched tissue samples were subsequently subjected to WGA using GenomiPhi (GE Healthcare) according to the manufacturer's instructions. All WGA samples were purified using a sodium-acetate-ethanol precipitation step as recommended by the manufacturer. The WGA-amplified DNA was quantified and normalized to 10 ng/µL as previously described. All WGA-amplified samples were genotyped as described above. A subset of 19 WGA hair samples (corresponding to the 19 individuals for which tissue was WGA-amplified) were genotyped twice more. Observed and expected heterozygosities, allele number, frequency and Hardy-Weinberg equilibrium were calculated using GENALEX version 6 (Peakall & Smouse 2006).

Statistical analyses were performed using SPSS version 12.01. Tissue, hair, WGA-tissue, WGA-hair and WGA-hair over three replicated genotyping attempts (henceforth WGA hair × 3) were treated as separate categories. A consensus genotype was obtained for WGA hair × 3 using the rules outlined in Table 1. The method used maximizes the chance of assigning a genotype as a heterozygote. This approach was used because previous genetic studies using samples such as hairs have found a high rate of allelic dropout (Taberlet *et al.* 1999). At each locus, we tested for differences between expected and observed heterozygosities, the number of alleles, the number of private alleles and genotyping success between each DNA category. Kruskal-Wallis tests with Monte Carlo resampling were used to ascertain significance for proportional data, including heterozygosity and genotyping success. Ten thousand tables were used for Monte Carlo resampling and ANOVA

Table 1 Rules for assigning consensus genotypes (WGA hair × 3)

Situation	Accept heterozygote	Accept homozygote	Accept amplified genotype	Assume heterozygote	Discard genotype
3 matching heterozygotes	Yes	×	×	×	×
3 matching homozygotes	×	Yes	×	×	×
2 matching heterozygotes 1 no amplification	Yes	×	×	×	×
2 matching homozygotes 1 no amplification	×	Yes	×	×	×
1 heterozygote, 2 homozygotes matching either heterozygote allele	Yes	×	×	×	×
2 matching heterozygotes, one concordant homozygote	Yes	×	×	×	×
2 nonamplifications, 1 heterozygote or homozygote	×	×	Yes	×	×
3 mismatching homozygotes	×	×	×	×	Yes
2 mismatching heterozygotes	×	×	×	×	Yes
2 different homozygotes with 1 no amplification	×	×	×	Yes	×

was used in addition to nonparametric tests. The allelic richness (number of alleles) across all loci was compared using ANOVA for differences between DNA sources. The number of private alleles was calculated in GENALEX version 6 (Peakall & Smouse 2006). The potential effects of sample size upon allelic richness were accounted for by applying a rarefaction technique (HP-Rare; Kalinowski 2004; Kalinowski 2005).

The percentages of allelic dropout, correction of allelic dropout, mismatching genotypes, concordant genotypes, no amplification and new amplification were analysed for each DNA source, for each locus genotyped (see Table 2). Concordance of genotypes was assessed by comparison between WGA substrate and the original substrate (e.g. genotypes from WGA hair were compared with those from nonamplified hair DNA) Results for hair-extracted DNA were compared to those for DNA from tissue. Because tissue was stored in optimum conditions and yielded high quantities of DNA, tissue genotypes were assumed to be correct over genotypes from all other DNA sources, unless there was amplification failure for that sample. We are confident that genotypes obtained from tissue are robust because we observed a low mismatch rate between mother and offspring genotypes obtained from tissue for the loci used (average error rate of 0.03 over all loci).

Results

Typical amounts of DNA obtained from hair samples post-WGA were 60 ng/μL. The maximum concentration obtained from WGA hair samples was 223 ng/μL, while some samples failed to amplify at all (see Fig. 1). We could not obtain accurate post-WGA concentrations from tissue because of the extremely high concentration of the majority of samples (over 450 ng/μL). The genotyping success rate varied significantly by DNA source (ANOVA $F_{4,15} = 5.226$, $P = 0.008$; nonparametric tests gave the same

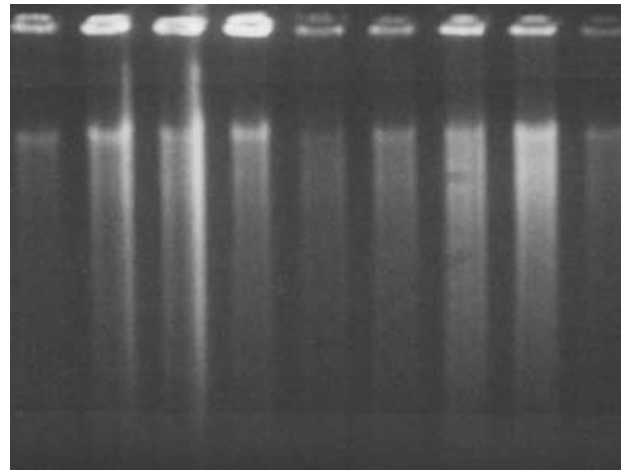


Fig. 1 An agarose gel showing post-WGA DNA from hair samples. The variable amplification achieved in different samples is clearly visible (possibly because of variation in the quality of the template DNA).

result). Tissue samples provided the highest genotyping success (86.1%), closely followed by hair (84.2%), WGA hair × 3 (84.0%) and WGA tissue (82.5%, Fig. 2a). The genotyping success rate for WGA hair (63.2%) was significantly lower than for other sources ($\chi^2 = 10.079$, $P = 0.018$, d.f. = 4). Genotyping success rates for DNA sources are shown in Table 2. There was no significant difference in expected heterozygosity between DNA sources across all loci ($\chi^2 = 2.513$, $P = 0.674$, d.f. = 4) (Fig. 3). The observed heterozygosity showed a nonsignificant trend of reduced heterozygosity in both WGA hair and WGA hair × 3 ($\chi^2 = 7.826$, $P = 0.083$, d.f. = 4). There were no significant differences in the average number of alleles between DNA sources across all loci (Fig. 2b), either with ($F_{4,15} = 0.641$, $P = 0.641$) or without rarefaction ($F_{4,15} = 0.902$, $P = 0.487$). Private alleles (Fig. 2b) were only present in hair and WGA

Table 2 Comparison of genotyping success across DNA sources

Locus	Comparison	Concordant genotypes (percentage)	Corrected dropout (percentage)	Caused dropout (percentage)	Mismatch (percentage)	No amplification (percentage)	New amplification (percentage)
Thu08	Tissue vs WGA tissue	79.0	0.0	10.5	0.0	10.5	0.0
	Tissue vs hair	47.9	0.0	16.7	2.1	25.0	8.3
	Tissue vs WGA hair	29.2	0.0	20.8	2.1	43.8	4.2
	Tissue vs WGA hair × 3	36.8	10.5	21.1	15.8	10.5	5.3
	Hair vs WGA hair	14.6	2.1	14.6	8.3	50.0	10.4
	Hair vs WGA hair × 3	21.1	15.8	21.1	15.8	10.5	15.8
Thu14	Tissue vs WGA tissue	42.1	0.0	52.6	0.0	5.3	0.0
	Tissue vs hair	41.7	2.1	22.9	8.3	12.5	12.5
	Tissue vs WGA hair	16.7	0.0	39.6	2.1	37.5	4.2
	Tissue vs WGA hair × 3	21.1	0.0	68.4	5.3	0.0	5.3
	Hair vs WGA hair	33.3	0.0	22.9	0.0	41.7	2.1
	Hair vs WGA hair × 3	52.6	0.0	31.6	15.8	0.0	0.0
Thu42	Tissue vs WGA tissue	63.2	0.0	0.0	0.0	31.6	5.3
	Tissue vs hair	70.8	0.0	0.0	0.0	14.6	14.6
	Tissue vs WGA hair	41.7	2.1	16.7	0.0	29.2	10.4
	Tissue vs WGA hair × 3	36.8	5.3	21.1	0.0	26.3	10.5
	Hair vs WGA hair	33.3	2.1	20.8	0.0	35.4	8.3
	Hair vs WGA hair × 3	36.8	5.3	26.3	0.0	26.3	5.3
Thu49	Tissue vs WGA tissue	78.9	0.0	5.3	0.0	0.0	15.8
	Tissue vs hair	79.2	2.1	4.2	0.0	2.1	12.5
	Tissue vs WGA hair	37.5	0.0	22.9	4.2	27.1	8.3
	Tissue vs WGA hair × 3	68.4	0.0	5.3	10.5	0.0	15.8
	Hair vs WGA hair	29.2	2.1	27.1	6.3	33.3	2.1
	Hair vs WGA hair × 3	84.2	0.0	5.3	10.5	0.0	0.0
Average	Tissue vs WGA tissue	65.8	0.0	17.1	0.0	11.8	5.3
	Tissue vs hair	59.9	1.1	10.9	2.6	13.6	12.0
	Tissue vs WGA hair	31.3	0.5	25.0	2.1	34.4	6.8
	Tissue vs WGA hair × 3	40.8	3.9	28.9	7.9	9.2	9.2
	Hair vs WGA hair	27.6	1.6	21.4	3.7	40.1	5.7
	Hair vs WGA hair × 3	48.7	5.3	21.1	10.5	9.2	5.3

The percentage of concordant genotypes was obtained from the total number of identical genotypes divided by the total number of genotypes, from the two DNA sources listed on the left of the table. The DNA source generating the accepted genotype is listed first. The same calculation was performed for each subsequent category. 'Corrected dropout' is a category describing the situation where the genotype of the first DNA source was a homozygote, and the compared source was a heterozygote, if the first DNA source was not tissue, and the tissue genotype was also a heterozygote. 'Caused dropout' describes the situation where the correct genotype was a heterozygote, and the compared genotype was a homozygote. 'Mismatch' describes the situation where the compared genotype contains one or more different alleles to the first genotype, where the first genotype was a heterozygote. 'No amplification' describes the situation where the compared DNA source failed to amplify. 'New amplification' describes the situation where the compared DNA source generated a genotype, but the first DNA source had failed to amplify.

hair and WGA hair × 3. Tests of Hardy–Weinberg equilibrium showed significant departures from equilibrium in WGA hair and WGA hair × 3 for different loci (WGA hair Thu49 $\chi^2 = 56.59$, $P = 0.001$, d.f. = 28; WGA hair × 3 Thu14 $\chi^2 = 49.65$, $P = 0.007$, d.f. = 28; Thu42 $\chi^2 = 23.53$, $P = 0.009$, d.f. = 10).

There was no significant difference in allelic dropout between DNA sources ($\chi^2 = 3.706$, $P = 0.629$, d.f. = 5). However, there were large differences between loci in most categories (see Table 2 for category definitions); for allelic dropout such differences were significant ($\chi^2 = 11.343$, $P = 0.005$, d.f. = 3), with Thu14 showing up to 68% allelic

dropout for a comparison of WGA hair × 3 with tissue. Over all loci, the best-performing WGA substrate was tissue. WGA tissue showed the highest percentage of concordant genotypes, and the lowest percentage of allelic dropout relative to non-WGA substrate (Table 2). WGA hair produced only 27% genotypes that were concordant with genotypes obtained from hair. A limited multiple-tubes approach improved this score (WGA hair × 3), but the percentage of concordant genotypes remained less than 50%. Despite these trends, differences in the percentage of concordant genotypes were not significant between DNA

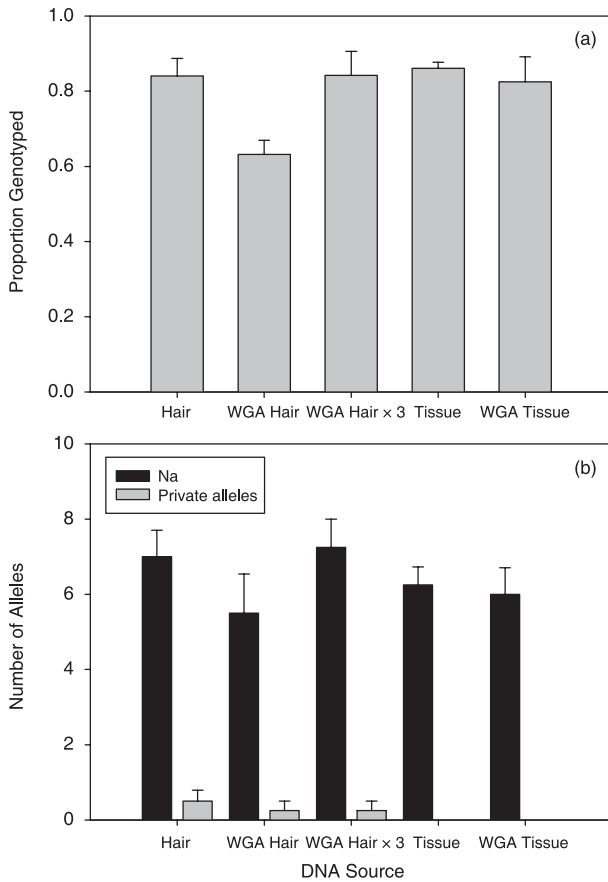


Fig. 2 (a) The proportion of samples successfully genotyped and (b) the average number of alleles per locus and the number of private alleles are shown \pm standard error bars for each DNA source.

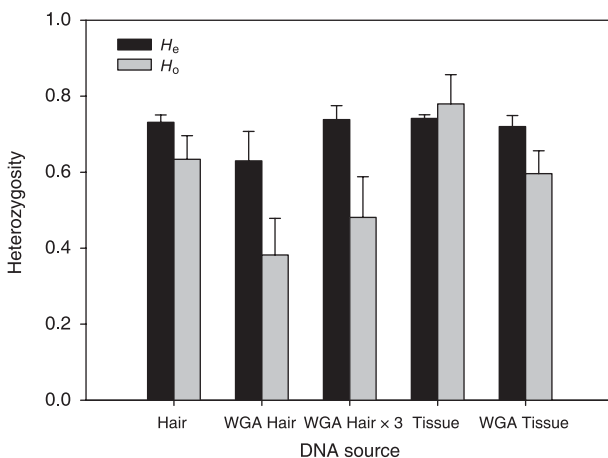


Fig. 3 The observed (H_o) and expected (H_e) heterozygosities across all loci. The average expected and observed heterozygosities for each DNA source is shown \pm standard error. The samples sizes for each source are as follows: Hair = 48, WGA hair = 48, WGA hair x 3 = 19, Tissue = 48, WGA tissue = 19.

sources ($\chi^2 = 8.643$, $P = 0.118$, d.f. = 5), or for comparisons between loci ($\chi^2 = 3.920$, $P = 0.283$, d.f. = 3).

Discussion

We have shown that the use of whole genome amplification of low-yield DNA substrate is beset with problems. Although DNA amplification was observed for both WGA hair and WGA tissue samples, amplification was greater for tissue samples. Since GenomiPhi preferentially amplifies unsheared DNA, this may indicate damage present in the hair samples. We observed problems with genotyping success rate, locus-specific allelic dropout and the generation of false alleles. Genotyping success was significantly poorer for WGA hair than for other DNA sources. Reduced genotyping success limits the utility of a DNA source for subsequent analyses, and may mean that insufficient data can be collected for a meaningful study. Our data suggest that this problem may be exacerbated when hair is the only available source of DNA. Excessive re-running of failed genotyping attempts may also incur unexpected costs. There was no significant difference in genotyping success between hair and tissue in this study. It should be noted that the routine genotyping of the tissue samples was conducted during a time when formamide used in the genotyping process was performing suboptimally (Hartnup & Gunn, unpublished data). This could partially account for a lower than expected genotyping success, but will only make our comparisons between hair and tissue more conservative. All hair and post-WGA tissue and hair samples were genotyped after any problems with the formamide had been resolved. Typical genotyping success rates for red squirrel tissue samples not included in this particular study reached an average of 95% across loci (Hartnup & Gunn, unpublished data).

The poor concordance of WGA tissue and tissue genotypes highlights the limitations of WGA even from high quality samples, particularly given the high allelic dropout rate (Table 2). Multiple PCRs post-WGA would be less of a logistical problem than pre-WGA, because of the increased amount of DNA available. Poor concordance between WGA tissue and tissue genotypes were due to either poor amplification or to allelic dropout, rather than to spurious alleles as in WGA hair. A multiple-tubes approach may be easier to interpret for WGA tissue than for WGA hair. We note that there were large differences between loci in allelic dropout rates and lack of amplification. This may be due to effects such as differential amplification in the WGA stage (discussed further below). Researchers with a large number of loci available could potentially maximize the success of WGA by doing pilot studies to determine the loci most likely to amplify well post-WGA.

Departures from Hardy–Weinberg equilibrium occurred only in WGA samples. There are a number of possible

explanations for the differences observed in dropout rates between loci. For example, different dropout rates may have been due to differential amplification of parts of the genome. Guanine-cytosine (GC) content, template length and secondary structure can all affect amplification of a given sequence (Lasken & Egholm 2003). Preferential amplification of unsheared DNA may also mean that if the locus is in a weak or easily sheared area of the genome, it will not amplify as well as loci in other positions.

Spurious private alleles detected in hair and WGA hair samples suggest the misamplification of alleles in hair-based or low quality DNA (Bradley & Vigilant 2002; Creel *et al.* 2003; Broquet & Petit 2004). Other studies have also found that low DNA template quantity can increase the number of spurious alleles (Foucault *et al.* 1996; Taberlet *et al.* 1996). It has been suggested that such false alleles may be due to slippage in the early stages of the PCR amplification process (Schlotterer & Tautz 1992; Goossens *et al.* 1998).

This study reiterates the importance of the appropriate storage of DNA sources. The hair used in this study was not stored in optimum conditions. The samples were kept at room temperature as opposed to methods that may better preserve DNA quality such as preservation in ethanol, silica desiccation or -20°C freezing (Roon *et al.* 2003). The hair samples were also kept in suboptimal conditions for many years. DNA is known to degrade significantly if kept for longer than 6 months even when stored in optimal conditions (Roon *et al.* 2003; Wandeler *et al.* 2003). With appropriate storage and quick sample processing times, noninvasive DNA sources have the potential to yield reliable genotyping, such as in the hairy nosed wombat *Lasiornhinus krefftii* (Sloane *et al.* 2000). Given that our DNA was obtained from poor quality sources, the method of WGA tested here may prove more effective as a tool for the extension of DNA supplies obtained from hair samples, or other poor quality sources of DNA, if they have been appropriately stored with short intervals between sample collection and processing. However, researchers should note that the method of WGA tested here was not foolproof even for WGA tissue. In addition, limited resources may further reduce the utility of whole genome amplification for many conservation or population genetic studies. The cost per reaction at the time of writing was over £2.00 for GenomiPhi. Although a number of companies now produce WGA kits, the list price of most WGA kits remains high enough to be prohibitive. We have attempted to use WGA techniques on degraded samples, which may reflect the condition of many precious samples. It has been suggested that Phi29 DNA polymerase is not suitable for amplifying degraded DNA (Holbrook *et al.* 2005). It is possible that other WGA kits, which do not use the Phi29 DNA polymerase, may be more suitable for this purpose, although these products are available at an increased cost. There is therefore a trade-off of cost vs. optimal genotyping.

We found that whole genome amplification was not suitable for hair samples even when a multiple-tubes approach was used. The present study used three replicated PCR experiments as a minimal multiple-tubes approach. Given the high rate of mismatching genotypes within each individual, it may not be possible to resolve an adequate number of genotypes at this level of replication. A more conservative approach to assigning consensus genotypes would require an increased number of replicates in order to obtain an acceptable level of genotyping success. The increased cost of multitubing in addition to the WGA enzymes may render this method inviable for many conservation studies. It appears that the technique of WGA tested here provides optimum performance with high-yield, appropriately stored DNA substrates such as tissue. This conclusion is in agreement with a recent study of the quantity of DNA substrate required for accurate WGA (Bergen *et al.* 2005), which found that both nonamplification and discordance of genotypes increased with decreased DNA input. If this method of WGA is to be used successfully for yield extension of DNA in conservation, we would urge potential users of this technique to make every effort to obtain fresh or optimally stored DNA sources and to use multiple PCRs to verify the genotypes of WGA DNA. The method of WGA used in this study is unsuitable for the recovery of DNA from old, degraded or low-yield samples.

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