

TECHNICAL ADVANCES

Searching for DNA in museum specimens: a comparison of sources in a mammal species

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Abstract

The number of genetic studies that use preserved specimens as sources of DNA has been steadily increasing during the last few years. Therefore, selecting the sources that are more likely to provide a suitable amount of DNA of enough quality to be amplified and at the minimum cost to the original specimen is an important step for future research. We have compared different types of tissue (hides vs. bones) from museum specimens of Iberian lynx and multiple alternative sources within each type (skin, footpad, footpad powder, claw, diaphysis, maxilloturbinal bone, mastoid process and canine) for DNA yield and probability of amplification of both mitochondrial and nuclear targets. Our results show that bone samples yield more and better DNA than hides, particularly from sources from skull, such as mastoid process and canines. However, claws offer an amplification success as high as bone sources, which makes them the preferred DNA source when no skeletal pieces have been preserved. Most importantly, these recommended sources can be sampled incurring minimal damage to the specimens while amplifying at a high success rate for both mitochondrial and microsatellite markers.

Keywords: historical samples, microsatellite DNA, mitochondrial DNA, museum specimens, real-time qPCR

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Introduction

Improvements in molecular techniques achieved during the last two decades have increased the variety of materials from which DNA can be successfully extracted. Biological collections around the world harbour a vast amount of specimens encompassing a wide taxonomic range. The possibility of extracting useful DNA from these specimens makes museums irreplaceable resources for the description and understanding of biodiversity through molecular systematics, phylogenetics and evolutionary studies. Furthermore, they provide access to historical intraspecific genetic patterns upon which changes in genetic variation in declining or invasive species can be gauged (Higuchi *et al.* 1984; Wandeler *et al.* 2007). The first molecular studies using mammal museum specimens were published in the mid-eighties and beginning of nineties (Higuchi *et al.* 1984; Thomas *et al.* 1990; Roy *et al.* 1994; Taylor *et al.* 1994), and the number of papers

using preserved specimens for molecular research has been steadily increasing since then.

Polymerase chain reaction (PCR) amplification success relies on the initial number of intact DNA templates. DNA quantity and quality in museum specimens mainly depend on the preservation treatments and the age of the samples (Wandeler *et al.* 2003, 2007). Inhibition is another major concern as different preservation techniques may result in the copurification of substances that inhibit the enzymes used to digest tissue or amplify DNA (Hall *et al.* 1997). Mitochondrial DNA (mtDNA) tends to be easier to amplify even after very long periods of storage and degradation simply because it is usually present in cells at higher copy numbers than nuclear DNA. Nevertheless, nuclear DNA has been also recovered from historical specimens in a remarkable number of studies (Miller & Waits 2003; Wandeler *et al.* 2003; Wisely *et al.* 2004; Hedmark & Ellegren 2005; Nystrom *et al.* 2006; Moraes-Barros & Morgante 2007; Morin *et al.* 2007).

Even though the age and the preservation treatment are out of geneticists' hands, we still have the opportunity to choose those sources that are most likely to

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provide a suitable quantity and quality of DNA to be amplified. When sampling museum specimens, one often has the opportunity to choose among different tissues, in some of which DNA may be better preserved than in others. Nevertheless, minimizing damage to the specimens must also be an important consideration (Rohland *et al.* 2004; Wisely *et al.* 2004). Therefore, it is critical to select the sources that are more likely to provide a suitable amount of DNA of enough quality to be amplified and at the minimum cost to the original specimen (Horváth *et al.* 2005).

In this study, we investigate which parts of museum specimens of mammals are optimal sources of DNA considering both DNA quantity and quality. We compare different sources for (i) mitochondrial and nuclear (microsatellites) DNA amplification success; and (ii) mitochondrial and nuclear DNA yield as estimated by real-time quantitative PCR (RT-qPCR).

Materials and methods

A total of 25 *Lynx pardinus* specimens from the mammalian collection at the Estación Biológica de Doñana – CSIC, Seville, Spain, for which both hide and skeleton materials were available, were used in this study (see Table S1 for further data information about specimens). The date of collection of these specimens ranged from 1954 to 2006. Eight samples were taken from each individual, four from hides [a piece of skin, a piece of footpad, footpad powder (footpad-p) and claw powder] and four from skeletal materials [diaphysis powder, maxillo-turbinal bone, mastoid process (MP) powder and canine powder]. Because all eight samples could not be taken from each specimen, the total number of samples used was 176, of which 25 were skin, 24 footpads, 25 footpad-p, 24 claws, 19 diaphysis, 21 maxilloturbinal bones, 20 MP and 18 canines. All powder samples were taken with a Dremel® bit tool (1.5–2 mm diameter). To avoid overheating, the instrument was kept at the minimum available speed setting (10 000–14 000 rpm) and continuous contact with sample was avoided (Flagstad *et al.* 2003). Canines were drilled from the root (Pichler *et al.* 2001; Pertoldi *et al.* 2005). Claws were drilled at their insertion point, where a blood coagulum is located (see Fig. S1 for detailed pictures).

DNA extractions

Hide samples, with the exception of claws, were pre-washed three times in 24 h with 1.5 mL of NTE solution (50 mM tris pH 9, 20 mM EDTA, 10 mM NaCl) (Johnson *et al.* 2004). DNA was extracted from all hide samples following a standard proteinase K/phenol-chloroform protocol, but alcohol precipitation was substituted by

ultrafiltration with Microcon® YM-30 (Higuchi *et al.* 1988). Proteinase K digestion was performed for 2 h at 56 °C followed by overnight incubation at RT. In each of the digestions, we added proteinase K to a final concentration of 2 mg/mL. Bone samples were digested and extracted using a silica-based protocol described in Rohland & Hofreiter 2007. In both cases, DTT (Dithiothreitol) was added to a final concentration of 50 mM to the digestion buffer. All extracts were eluted in a final volume of 100 µL and kept at –20 °C until used.

We made all extractions and reagent preparations in a low-copy number DNA laboratory, physically isolated from modern DNA laboratories and post-PCR laboratories. The laboratory was kept illuminated with UV lamps while not in use. Potential contamination was monitored by using several extraction blanks carried through all extraction steps and by including PCR blanks in all amplification reactions. We cleaned all equipment and surfaces with DNAZap™ or 20% dilution of commercial bleach and distilled water.

Real-time quantitative PCR

A 186-bp fragment of mitochondrial ATP8 amplified with primers: F: 3'-TGGGAGCTTAGACCTCTCCTT-5' and R: 3'-TTTTTCTCAAGGATTAAGTTGTTTTG-5' was used for assessing mtDNA yield by RT-qPCR. Primers F: 3'-GCATACCTGACTTTAATACA-5' and R: 3'-CAAAGCCACATTCTCTACAT-5', were used to amplify a 225bp ZFX/Y fragment for the estimation of nuclear DNA yield. Both sets of primers were designed to hinder human DNA amplification, a highly likely contaminant in museum specimens (Wandeler *et al.* 2003; Rohland *et al.* 2004). Human ATP8 and ZFX/Y amplification were tested and the results were negative—no amplification product was obtained when using human DNA as template.

Standards for RT-qPCR were made from high quality Iberian lynx extracts. Initial extract concentration was measured with Nanodrop® (ND-1000 Spectrophotometer) and dilutions were made as necessary to obtain the desired DNA concentration ranges (6×10^{-6} – $3 \text{ ng}/\mu\text{L}$ for ATP8 and 6×10^{-3} – $6 \text{ ng}/\mu\text{L}$ for ZFX/Y assays). Each standard dilution was replicated three times within each assay, while extracts were replicated twice. PCR blanks were added in all assays. We used a single preparation of the PCR reagent mix for all extracts, standards and controls. Because our standards are based on total DNA concentration, they cannot be transformed to copy numbers, but this should not be a problem if only differences among sources are to be characterized.

Amplification reactions contained 0.8% of bovine serum albumin (BSA) (20 mg/mL), 1 µM of each primer, 0.5x of QuantiTec SYBR Green PCR Kit (Qiagen) and

2 µL of DNA extract in a final volume of 25 µL. Cycling conditions for ATP8 were 15-min predenaturation step at 95 °C, followed by 40 cycles of amplification with 15 s at 94 °C, 15 s at 58 °C and 30 s at 72 °C. A final dissociation curve was also performed in order to check for primer dimer and other PCR artefacts: 1 min at 95 °C followed by 30 s at 55 °C and increasing up to 85 °C for 30 s. In the case of ZFX/Y, cycling conditions were the same but with an annealing temperature of 56 °C. RT-qPCR was carried out in an Mx-3005P cyler (Stratagene). Data were analysed with software MxPro v4.00 (Stratagene). Briefly, this software calculates unknown concentrations using the threshold cycle (C_t) of each well and the standard curve relating C_t and the logarithm of concentrations. Standard curves are calculated with a least mean squares approximation. The results from RT-qPCR were not biased by the amplification of artefacts or primer dimer as we only observed ATP8 or ZFX/Y products in the dissociation curve results.

Microsatellite amplification

As microsatellites are the most common neutral nuclear marker used in genetic diversity studies, we tested microsatellite amplification to validate amplification success from our tested sources. The test was based on amplification of Lyp82 microsatellite marker, a redesign of cat microsatellite Fca82 (Menotti-Raymond *et al.* 1999) based on Iberian lynx sequences, with primers F: 5'-TCACCGCTTAAGAAGAGGCTA-3' and R: 5'-TGAAGCTTCCGAAATGAGG-3'. Allele sizes in contemporary Iberian lynx range from 177 to 185 bp.

Amplification reactions contained 1x PCR Buffer, 2 mM MgCl₂, 0.25 mM dNTPs, 0.8% BSA, 0.4 µM of each primer, 0.4 U/µL BioTaq DNA Polymerase (Bioline) and 4 µL of DNA template in a final volume of 20 µL. PCRs were performed in a DNA Engine (PTC-200) Peltier Thermal Cycler (BIO-RAD) or biometra T-gradient cyclers (Biometra biomedizinische Analytik GmbH). Cycling conditions were 2-min at 94 °C followed by 40 cycles of amplification with 30 s at 92 °C, 30 s at 55 °C and 30 s at 72 °C, and a final 5-min extension step at 72 °C. Amplification success was checked in a 1.5% agarose gel with SyberSafe DNA gel stain (Invitrogen).

Data analysis

We used generalized linear mixed models (GLMMs) to compare the probability of amplification (for ATP8, ZFX/Y and Lyp82) and the concentration of DNA (for ATP8 and ZFX/Y) for different sources (see Table S1, for detailed data). Note that amplification probabilities of ATP8 and ZFX/Y were based on positive amplification in RT-qPCR. In the first set of analyses, we searched for

differences between tissues, i.e. hide vs. bone samples. In a second set of analyses, we considered all sources as independent levels. Canine samples were classified as bones samples because they are hard tissue, and thus more similar to bone than to hides, and because we used the same extraction protocol as for bone samples. As sample weight and age of the samples affect the quantity and amplification success (Wandeler *et al.* 2003), we included them in the analyses to control for these effects.

When analysing amplification probability, GLMMs were run using a binomial distribution of data and a logit link function. Weight, age and tissue/source were used as fixed effects. Specimen was used as a random effect in order to remove any variation associated with individual specimens. For the analysis of DNA concentration, we used a Poisson distribution with a log link function as it provided the best fit to the data; extracts that did not amplify were excluded. Skin and footpad levels had to be excluded from the analysis of ZFX/Y amplification success because no positive results were obtained. Furthermore, all extracts from hides were excluded from ZFX/Y concentration analysis because too few data remained to obtain acceptable models. We used SAS 9.1.3 Service Pack 2 to perform the GLMM analyses.

Inhibition test

As we observed possible indications of inhibition (i.e. digestions that did not perform well), we tested inhibition by mixing working extracts with those which we suspect were inhibited (test extracts). The assay was the same as for Lyp82 amplification but with the addition of 4 µL of the test extract and the removal of 4 µL of water to maintain reagents' concentration. For the working extracts, we used one blood extract and two museum extracts that consistently amplified for Lyp82. As test extracts, we used two footpads, two footpad-p and two skin extracts, all of which failed to amplify for all markers. We tested all combinations of working extracts with the test extracts, and we made three PCR replicates in order to avoid false positive inhibitions due to PCR failures. We checked amplification success in a 2% agarose gel.

Results

We analyzed a total of 172 extracts corresponding to two types of tissues [hide ($n = 94$) and bones ($n = 78$)] and eight types of sources. Four hide samples (two skins and two footpads) could not be used because they were not digested by proteinase K.

The probability of amplification was consistently higher for bones than for hides, either for ATP8, ZFX/Y or Lyp82 (Table 1; Fig. 1). If we consider all sources

Table 1 GLMM results of tissue and source effects on concentration and probability of amplification of ATP8, ZFX/Y and the microsatellite Lyp82

	Probability of amplification	Concentration
ATP8		
Tissue	22.94 (1)***	11.13 (1)**
Weight	5.26 (1)*	4.06 (1)*
Age	1.35 (1)	3.64 (1)
	<i>N</i> = 172	<i>N</i> = 124
Source	6.36 (7)***	3.65 (7)**
Weight	1.52 (1)	4.92 (1)*
Age	1.15 (1)	4.84 (1)*
	<i>N</i> = 172	<i>N</i> = 124
ZFX/Y		
Tissue	14.80 (1)***	2.01 (1)
Weight	0.05 (1)	0.84 (1)
Age	13.63 (1)***	4.84 (1)*
	<i>N</i> = 172	<i>N</i> = 26
Source	2.14 (5)	3.56 (3)
Weight	0.01 (1)	—
Age	14.03 (1)***	—
	<i>N</i> = 127	<i>N</i> = 22
Lyp82		
Tissue	14.64 (1)***	—
Weight	2.17 (1)	—
Age	2.20 (1)	—
	<i>N</i> = 172	
Source	5.54 (7)***	—
Weight	0.18 (1)	—
Age	1.52 (1)	—
	<i>N</i> = 172	

F-value, degrees of freedom in parentheses, significance with asterisks and the sample size for each analysis (*N*) are shown in the table. Results are shown for both tissue (bones vs. hides) and source (skin, footpad, footpad-p, claw, diaphysis, maxilloturbinal bones, MP and canines) analyses, and probability of amplification and concentration analyses. The model corresponding to the effect of source on ZFX/Y concentration was run considering source as the only fixed effects as there were too few data to obtain reliable models when including weight and age. We previously evaluated weight and age effects on this response term and we found no effect ($P = 0.9881$ and $P = 0.0701$ respectively). Because there were almost no positive results within hide levels, only bone levels were considered in ZFX/Y concentration analysis. In the case of microsatellite Lyp82, we only evaluated probability of amplification. MP source was the only one with one hundred per cent of amplification of ATP8 and Lyp82, but we introduced one negative amplification value in order to perform GLMM analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

separately (Table 1; Fig. 1), all bone sources continue to have the highest amplification success. MP was the only material with 100% amplification success for both ATP8 and microsatellite Lyp82. Interestingly, claws are the best among alternative hide sources, yielding as high amplification success as bone sources (Fig. 1). DNA concentration data show a pattern similar to amplification success

(Table 1; Fig. 1), with bones yielding more DNA than hides. However, claws have lower concentrations of nuclear DNA than bone sources despite showing similar amplification success. As observed in Table 1, weight and age have significant effects on both probability of amplification and concentration in some cases; showing the need to control for their effects.

All these results could potentially be affected by PCR inhibition due to copurified substances that are present in preserved specimens. Our inhibition test shows that there are clearly inhibitors in at least some of the extracts. Skin extracts inhibited the PCRs for all three replicates of the control PCRs. In contrast footpad or footpad-p extracts did not show any sign of inhibition, with the only exception of one of the two footpad-p extracts, which appeared to decrease the amplification yield of one of the working museum extracts.

Differences in efficiency between RT-qPCR and standard PCR, in probability of amplification of different size products and in efficiency between PCR primers prevent the comparison among the different marker amplification rates or DNA yields. The expected higher performance of multiple copy mtDNA markers over single-copy nuclear markers was generally observed, although in a few cases ($n = 18$) we obtained amplification of Lyp82 marker despite failed amplification of the ATP8 marker. Most of these cases involved footpad or footpad-p extracts ($n = 15$), probably indicating a lower ratio of mtDNA to nuclear content in this tissue. On the other hand the consistently higher amplification probability observed for Lyp82 vs. ZFX/Y might be attributed to its shorter amplification product, the lower amplification efficiency of RT-qPCR, or to more efficient primers.

Discussion

With the used extraction methods, our results show that bone samples are to be preferred when possible and that skull samples, especially MP, seem to be the source most likely to yield good quality DNA for PCR amplification, even for low copy number targets such as nuclear microsatellites. In contrast, hide samples are less prone to success, likely because of degradation and inhibition due to preservation substances commonly used in pelt preparation.

The poor performance of hide samples may in part be attributed to inhibition problems. We observed some preliminary indications of inhibition from failures in proteinase K digestion that were later confirmed in test PCR reactions for some extracts. The inhibition test demonstrated that there are inhibitors in hide extracts, although we cannot discard insufficient DNA yield in these or other hide extracts due to a poor DNA preservation. Moreover, our results indicate that inhibition problems

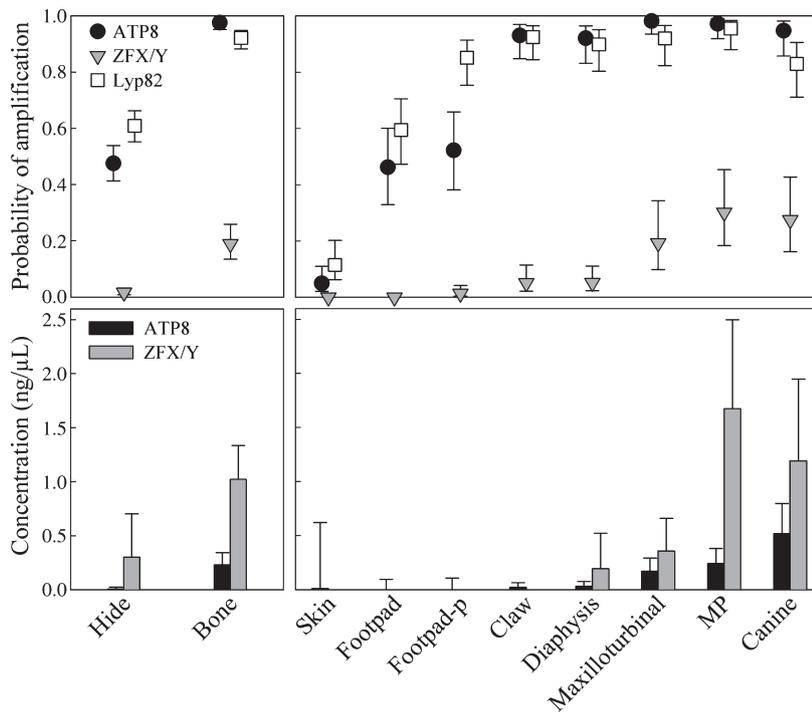


Fig. 1 Mean amplification probabilities and mean DNA concentrations obtained from different tissues or sources. Mean values and standard errors refer to the last squares means given by models when an average weight, average time and average specimen are considered. Tissue analyses are on the left, source analyses on the right. Bones have a higher success than hides in both tissue and source analyses. However, claw samples present a probability of amplification of microsatellite Lyp82 as high as bone sources despite having lower DNA concentration. In contrast to footpad, footpad-p source has also amplification rates similar to those of bones for Lyp82. Note that probability of amplification is based on standard PCR and product detection in agarose gel for Lyp82, while based on RT-qPCR results for ATP8 and ZFX/Y.

were not completely prevented by the final ultrafiltration step or the addition of high concentrations of BSA in the PCR, methods that have previously been shown to effectively prevent inhibition by some, but not all inhibitory substances (Yang *et al.* 1997). Despite the generalized inhibitory problems we have found, our results show that claw samples are the best DNA source from hides, what suggests that keratin may hamper the entrance of damaging and inhibiting agents. Furthermore, DNA extraction from claw coagula has the practical advantage of not entailing extensive and long washing steps. Similarly, and in contrast to hides, bone structure offers a safer environment for DNA conservation, since light, oxygen and other damaging factors may not reach the inner tissue (Cooper 1994). Moreover, bones are subjected to different conservation treatments than hide pelts. While bones are usually boiled, skins are treated with enzymatic reagents that can degrade DNA and inhibit digestion and PCR enzyme reactions.

Although our results come from the analyses of specimens from a single species, the Iberian lynx, we are certain that they will prove useful for studies of other carnivores. This is very likely for other mammals as well and maybe even for vertebrates in general. We demonstrate that bones are more likely to contain amplifiable DNA than hides, a result that likely applies to any vertebrate species since museum preservation treatments are the same for all these specimens and little intrinsic differences are expected among different vertebrates. In the case of hides, we demonstrate that it is better to look for

alternatives to skin that might hamper the entrance of inhibitors and damaging agents such as claws as illustrated in this study. Similarly, a blood clot within the feather calamus has been also shown to be a better DNA source than the tip of the calamus or the skin in birds (Horváth *et al.* 2005). Importantly, results from a number of previous studies that used museum specimens seem to support our general findings (Miller & Waits 2003; Wandeler *et al.* 2003; Wisely *et al.* 2004; Hedmark & Ellegren 2005; Nystrom *et al.* 2006; Moraes-Barros & Morgante 2007; Morin *et al.* 2007).

The results we present should provide encouragement for more scientists to use museum specimens in genetic research and to test their validity in a broader range of species. Lastly, the present study highlights once again the problems that museum preparation techniques pose for DNA preservation and use. Thankfully, many museums around the world are expanding their facilities for storing fresh tissues with the specific aim of facilitating future genetic analyses. Yet, traditional specimens will remain an important, and in many cases irreplaceable, source of biological—including genetic—information.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1 Specimens from which samples were taken with the Dremel® tool that show the low impact of our sampling methods.

Table S1 Data used in GLMM analyses

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