ISSN NO. 2320-5407

Journal homepage:http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Digging out treasure from museum specimen: A comparison of extraction methods and PCR polymerase enzymes for DNA from old taxidermy samples.

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Manuscript Info *Abstract*

Manuscript History:

Received: 15 July 2014
Final Accepted: 29 August 2014
Published Online: September 2014

Key words:
Phylogenetic; extraction; molecular ecology; taxidermy samples; fragmented DNA

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Museums are treasure-houses of samples from rare and threatened species. These samples are used in various studies such as phylogenetic and molecular ecology studies. They are also used to obtain information required for wildlife forensic cases. In the present study, the use of various commercially available kits and the traditional phenol/chloroform DNA extraction protocol were compared. They were compared in terms of the purity and yield of DNA and success of PCR amplification. Two mitochondrial genes, 12s rRNA and cytochrome b, from three mammal species (*Ratufa macroura*, *Petaurista petaurista albiventer*, *Felis chaus*) were used. No statistical significant differences were found between the extraction methods. DNA purity rather than yield was found to be strongly correlated with PCR success. It is concluded that research work on fragmented DNA from old taxidermic samples should aim at extracting pure DNA with good DNA yield and at amplifying short target regions (400–500 bp) to obtain the maximal amplification rate.

Introduction

Molecular studies are very useful in phylogenetics, resolving taxonomic problems, differentiation of hybrid populations, providing information about the behavior and status of species, determining the status of species, comparing past and present diversity, detecting possible losses of genetic diversity (Baker 1994; Weber *et al* 2000) and wildlife forensics.

Now a days, museum around the world are receiving increasing number of request for tissue or bone samples from preserved specimens for molecular studies. The ease of use of molecular technique, coupled with demand for broad taxon sampling, encourages many researchers to study specimens of museum collection, particularly when phylogenetic analysis of rare or extinct species needs to be carried out, extant and historical populations are to be compared or wildlife forensic investigations are to be conducted (Haude and Braun 1988; Cooper 1994; Cooper 1997; Sheldon 2001; Willerslev and Cooper 2005; Wandeler *et al* 2007; Kruckenhauser and Haring 2010; Payne and Sorenson 2002). The ideal materials for obtaining DNA samples are frozen or ethanol-preserved tissue samples. Unfortunately, the most readily available materials are bones and taxidermic specimens, prepared as stuffed or flattened skins. Nowadays, several well known protocols are used to obtain and amplify DNA segments from museum specimens (Shedlock *et al* 1997; Yang *et al* 1997; Pichler *et al* 2001; Junqueira *et al* 2002), but these protocols require the use of commercial kits and special reagents that are prohibitively costly for many researchers.

In the absence of frozen or ethanol-preserved tissue samples, taxidermic skins may be the only source of DNA. In this paper we compare the use of various commercially available DNA extraction systems and their performance in terms of purity and yield of DNA from taxidermy samples and success of PCR amplification.

Arsenic is used in taxidermy because of its insect-repellent effect. Usually, a 10% arsenic solution is brushed on the inside of a skin when it is being prepared, or arsenic is distributed as a powder in the fur. There are health concerns associated with the use of arsenic, and so this has been banned in many vertebrate collections (Marte

et al 2006). Arsenic has a clear negative effect on PCR performance above a threshold of 5.4 µg/µl (Topfer *et al* 2011).

Materials and Methods

We used taxidermic samples of two species of the family Sciuridae, i.e. *Ratufa macroura* and *Petaurista petaurista albiventer* (Mammalia: Rodentia) and one species of the family Felidae i.e. *Felis chaus* (Mammalia: Carnivora). These samples were from the mammals section of the Zoological Survey of India, Kolkata. The age of the specimens ranged from 26 to 90 years (Table 1). All the tools used were flame-sterilized initially and before each time that they were used on a new specimen. A small fragment of taxidermic skin (approximately 2 cm × 0.5 cm) was sliced from the specimen such that there was no significant loss of skin and further studies would not be compromised. All skin fragments were collected in sterile 2 ml centrifuge tube (Barros and Morgante 2007).

DNA isolation: DNA isolation of the 6 samples from species *Ratufa macroura*, *Petaurista petaurista albiventer*, *Felis chaus* was carried out using (a) commercially available kits (Invitrogen, Nucleo-pore, MERCK, HIMEDIA) and (b) the traditional phenol/chloroform extraction method. DNA isolation reactions were carried out in a sterile environment. All plastic materials were exposed to UV light, and all surfaces were wiped with 70% (v/v) ethanol. Fur was removed from skin samples using a sterile scalpel, after which the samples were washed with sterile milliQ water and ethanol 70% (v/v). Each sample was hydrated before digestion by incubation in 1 ml of TE solution (10mMTris and 1mM EDTA; pH 7.6) for 24 hours (Barros and Morgante 2007). DNA isolation was performed using each isolation kits according to the respective manufacturer's protocol. Another set of hydrated samples was digested in 10mMTris–HCl, pH 8.0/ 100mMNaCl/ 1 mM EDTA/ 0.5% SDS/ 50 mM DTT/ proteinase K (0.5 µg/ml) for 10–12 hours at 50°C. The DNA was purified by extraction with phenol twice, with phenol/chloroform (1:1 (v/v)) once, and with chloroform once. The DNA was precipitated with 0.1 volume of 3M sodium acetate (pH 5.3) and 2.5 volumes of ice-cold absolute ethanol. The quantity of the DNA was determined using a Qubit II fluorometer (Invitrogen) and the purity of the DNA was checked using a biophotometer (Eppendorf).

PCR amplification: The DNA samples were divided into two categories: samples with low DNA concentrations (<100ng/ml) and those with high DNA concentrations (>1µg/ml). All the samples with high DNA concentration were diluted to a concentration of 1ng/µl. The samples were then used for amplification of 12s rRNA and cytochrome b using four different types of DNA polymerase [Platinum Taq DNA Polymerase (Invitrogen), Accuzyme™ DNA Polymerase (BIOLINE), GeNei™Taq DNA Polymerase (MERCK), Thermo Scientific Dream Taq DNA Polymerase (Thermo Scientific)] in order to select the best-performing enzyme for further experiments. The sequence of primers used for amplification was as follows: 12s rRNA, L1091 (5′-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3′) and H1478 (5′-TGACTGCAGAGGGTGACGGGCGGTGTGT-3′); cytochrome b, L14841 (5′-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3′) and H15149 (5′-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3′) (Kocher *et al* 1989). The PCR reaction was performed in a Q-cycler (Quanta Biotech) in 10 µl (total volume) of reaction mixture [(10× PCR buffer (with $MgCl_2$), 1 µl; 10 mM dNTPs, 1µl; 5pmol primer, 0.36µl; 1U Taq enzyme; template, 4µl (samples with low DNA concentration) & 1µl (samples with high DNA concentration)]. The polymerase chain reaction for 12s rRNA & cyt b gene consisted of initial denaturation at 94°C for 4 minutes, a cycle of denaturation at 94°C for 1 minute, hybridization at 55°C (for 12s rRNA) and 50°C (for cytochrome b) for 1 minute, and extension at 72°C for 2 minutes, followed by final elongation at 72°C for 10 minutes. The cycle was repeated 35 times.

Electrophoresis of 5µl of the amplified mixture was carried out in a 2% agarose gel in TBE buffer (89mM Tris, 89mM boric acid, 2mM EDTA; pH 8.0) with ethidium bromide (0.5µg/ml).

Results

DNA yield and purity: The five isolation methods used in the study does not show statistical significant differences in terms of mean DNA yield (Friedman's Rank Test, χ^2=8.235, p=0.083) and mean DNA purity (Friedman's Rank Test, χ^2=8.504, p=0.075). However, the better DNA yield was obtained using the GeneiPure™ mammalian genomic DNA purification kit (MERCK, 36.79µg/ml), followed by the phenol/chloroform extraction method (13.37µg/ml). The DNASure Tissue Mini Kit (Nucleo-pore) gave the lowest mean DNA yield (5.29µg/ml) (Table 2). The GeneiPure™ mammalian genomic DNA purification kit, MERCK gave the maximum mean purity (1.72), with 66.6% of the samples having pure DNA, followed by the DNASure Tissue Mini Kit (Nucleo-pore, 1.57), with 50% samples having pure DNA. The least mean DNA purity was obtained using the phenol/chloroform extraction method (1.27), with none of the samples having pure DNA (Table 2).

Table 1. Detail of samples used in the present study.

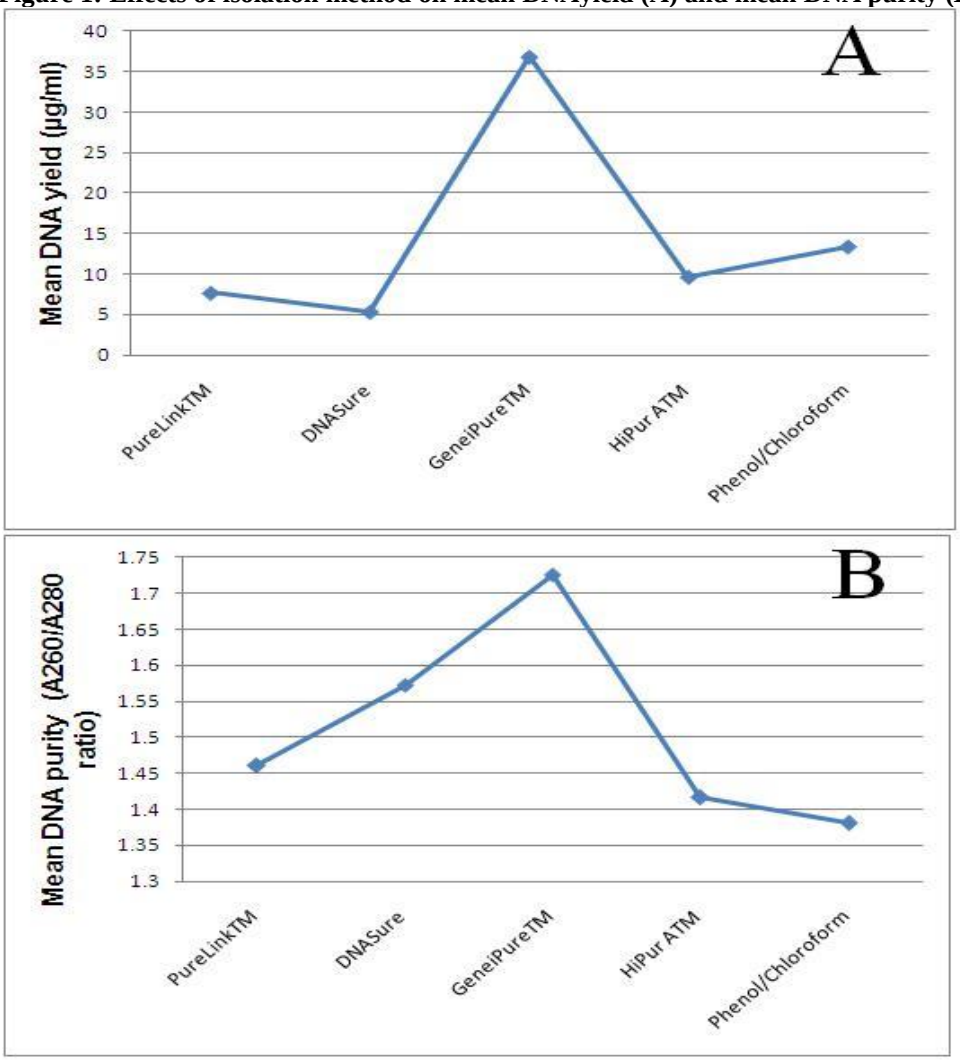
Species	Sample	Collection Date	Sex	Collected From	Registration number
Ratufa macroura (Pennant)	RM-O	Oct' 1930	♀	Dharampuri Range, Tamil Nadu, India	15131
	RM-N	Oct' 1950	♂	Dharampuri Range, Tamil Nadu, India	15173
Petaurista petaurista albiventer (Pallas)	PA-O	April' 1922	♂	Dharmasala, Punjab, India	15052
	PA-N	Nov' 1958	♀	Naggur, Punjab, India	15373
Felis chaus (Schreber)	FC-O	June' 1972	♂	Karaput, Orissa, India	20865
	FC-N	Sep' 1987	♂	Puri, Orissa, India	23956

Table 2. Effect of different isolation method on DNA yield and purity.

Protocol Used	Supplier (catalog No.)	Mean DNA yield (µg/ml)	Mean DNA purity (A_{260}/A_{280} ratio)	No. of samples with pure DNA[1] (%)
PureLink™Genomic DNA Mini Kit	Invitrogen (K1820-00)	7.641	1.46	2 (33.3)
DNASure Tissue Mini Kit	Nucleo-pore (NP-61305)	5.29	**1.572**	**3 (50)**
GeneiPure™Mammalian Genomic DNA purification Kit	MERCK(612115500021730)	**36.79**	**1.726**	**4 (66.6)**
HiPur A™ Forensic Sample Genomic DNA Purification Kit	HIMEDIA(MB524)	9.62	1.416	2 (33.3)
Phenol/Chloroform Extraction	Not Applicable	**13.37**	1.38	0 (00)

[1]**samples with A_{260}/A_{280} ratio 1.7-2.0 were considered pure.**

Figure 1: Effects of isolation method on mean DNAyield (A) and mean DNA purity (B).



PCR amplification success: All the isolated DNA samples were amplified for 12s rRNA and cytochrome b. There was no statistical significant differences in mean DNA yield (Mann-Whitney Test, Z=0.00, *p*=1.00) and in mean DNA purity (Mann-Whitney Test, Z= -1.732, *p*=0.083) with PCR success and nonsuccess (extraction methods that gave more than 40% of positive amplicons were considered as PCR success). Though, the DNA isolation method with the best overall PCR success, i.e. maximum number of positive amplicons, was from the one using the DNASure Tissue Mini Kit (Nucleo-pore; 54.2% and 62.5% positive amplicons of 12s rRNA and cyt b, respectively), followed by the method using the GeneiPure™ mammalian genomic DNA purification kit (MERCK, 50% and 50% positive amplicons of 12s rRNA and cyt b, respectively) (Figure 2). The DNA isolation method with the least overall PCR success was the phenol/chloroform extraction method (37.5% and 33.3% positive amplicons of 12s rRNA and cyt b gene, respectively) (Table 3).

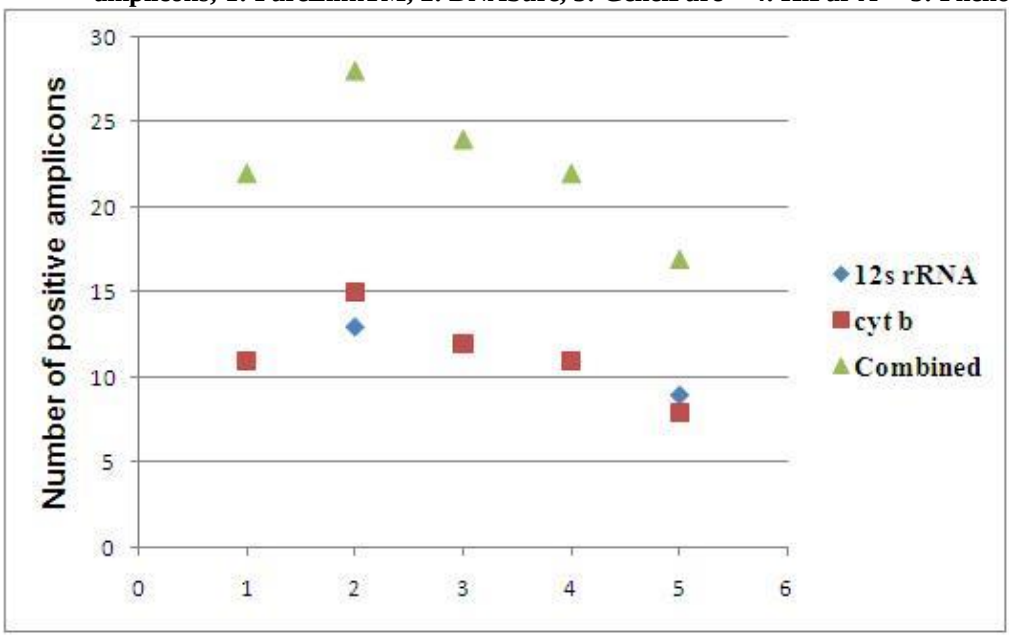
Sequencing of amplicons and BLAST searches confirmed the identity of all the tested samples, indicating that contamination had no effect on our PCR results. One sequence of the 12s rRNA (434 bp) of *Ratufa macroura* was submitted to the NCBI database, with accession no. KJ417131.

Table 3. Effect of different isolation method on PCR amplification as measured by number of positive amplicons.

Protocol Used	Number of positive amplicons[1] (%)		
	12s rRNA	cyt b	Combined
PureLink™ Genomic DNA Mini Kit	11(45.8)	11 (45.8)	22 (45.8)
DNASure Tissue Mini Kit	**13 (54.2)**	**15 (62.5)**	**28 (58.3)**
GeneiPure™ Mammalian Genomic DNA purification Kit	12 (50)	12 (50)	24 (50)
HiPur A™ Forensic Sample Genomic DNA Purification Kit	11 (45.8)	11 (45.8)	22 (45.8)
Phenol/Chloroform Extraction	09 (37.5)	08 (33.3)	17 (35.4)

[1]**defined as number of positive bands observed.**

Figure 2: Effects of isolation method on PCR amplification as measured by the number of positive amplicons; 1: PureLinkTM, 2: DNASure, 3: GeneiPure™ 4: HiPur A™ 5: Phenol/Chloroform.



DNA polymerase activity: Four different DNA polymerase enzymes were studied for their activity according to their amplification success with samples with low DNA concentrations (<100ng/ml) and samples with high DNA concentration (>1µg/ml). With samples with low DNA concentrations, the proportion of positive amplicons obtained using Accuzyme™ DNA Polymerase (BIOLINE) was 70%. The next highest proportion (50%) was obtained using GeNei™ Taq DNA Polymerase (MERCK) and Dream Taq DNA Polymerase (Thermo Scientific). The lowest proportion of positive amplicons (35%) was obtained using Platinum Taq DNA Polymerase (Invitrogen) With samples with high DNA concentrations, the greatest proportion of positive amplicons (57.5%,) was obtained using Platinum Taq DNA Polymerase (Invitrogen) and GeNei™Taq DNA Polymerase (MERCK), followed by Dream Taq DNA Polymerase (Thermo Scientific, 37.5%). With high DNA concentrations, the lowest proportion of positive amplicons (27.5%) was obtained using Accuzyme™DNA polymerase (BIOLINE, Table 4).

Table 4. Amplification success of different DNA polymerase enzyme on low DNA concentration (<100ng/ml) & high DNA concentration (>2µg/ml).

DNA Polymerase Used	Supplier/Cat. No	Positive amplicons (%)	
		In low DNA concentration (<100ng/ml)	In high DNA concentration (>1µg/ml)
Platinum Taq DNA polymerase	Invitrogen (10966-018)	7 (35)	**23 (57.5)**
Accuzyme™DNA polymerase	BIOLINE (BIO-21051)	**14 (70)**	11 (27.5)
GeNei™Taq DNA polymerase	MERCK (610601600031730)	10 (50)	**23 (57.5)**
Dream Taq DNA polymerase	Thermo Scientific (EP0702)	10 (50)	15 37.5)

Discussion

DNA yield and purity: The present work is to compare the use of different DNA isolation methods on old taxidermic samples. Work of this kind is important for diverse molecular studies (phylogenetic studies, molecular ecology, and wildlife forensics). Obtaining a good concentration of DNA without compromising the purity is needed in these studies. The need for DNA purity, i.e. contamination-free DNA, in ancient DNA studies has been acknowledged, and several studies have focused on developing extraction protocols optimizing not only yield but also purity (Ho¨ss and Pa¨a¨bo 1993; Ha¨nni *et al* 1995; Kalmar *et al* 2000; Rohland and Hofreiter 2007). Chemicals like arsenic and sodium chloride are used in taxidermy. These substances can interfere with the PCR even when the DNA yield is high.

It was found in the present study that the GeneiPure™ mammalian genomic DNA purification kit (MERCK) and DNASure Tissue Mini Kit (Nucleo-pore), with mean DNA purities of 1.72 and 1.57, respectively, can be used for obtaining DNA of high yields and purity from taxidermic samples. Though, the five isolation methods used in the study does not show statistical significant differences in terms of mean DNA yield and mean DNA purity because of less number of taxidermy samples studied in this study. It was also found that hydrating taxidermic samples in 1× TE buffer for 24 hours enhances the DNA yield. Hydrated tissues are digested faster compared with skin samples that are not hydrated in TE buffer (Barros and Morgante 2007).

PCR success: The present study had shown no statistical significance results, but by comparing the 'p' value of mean DNA yield (p=1.00) and mean DNA purity (p=0.083), mean DNA purity is better showing the relationship with PCR success. This study confirms that the choice of DNA polymerase enzyme affects the success of the PCR, as reported previously (Telle and Thines 2008; Rohland and Hofreiter 2007). This is not only due to differences in the structure and catalytic properties of the enzymes but also due to differences in enzyme purity and polymerase buffer chemistry (Telle and Thines 2008; Rohland and Hofreiter 2007; Pa¨a¨bo 1990). It was found that Accuzyme™DNA Polymerase (BIOLINE) was most effective in amplifying the target genes even with low DNA concentrations (<100 ng/ml). It was also found that with high DNA concentrations, Platinum Taq DNA Polymerase (Invitrogen) and GeNei™Taq DNA Polymerase (MERCK) had the highest amplification success rates (Table 4).

Fragmented DNA in old taxidermic samples: Old (26 to 90 years old) taxidermic samples were used in the present study. When the amplification of cytochrome b gene was carried out with same PCR condition mentioned above but using different set of primers that target approximately 1,040 bp region (Oshida *et al*, 2000) L14724 5'-GATATGAAAAACCATCGTTG-3' and H15910 5'-GATTTTTGGTTTACAAGACCGAG-3', then no PCR product was observed. This can be concluded that the DNA isolated in this study was fragmented and thus target region for the Oshida *et al* cytochrome b primer might have been missing in the DNA extracts for the PCR. Short fragments are known to be abundant in taxidermic samples. The profusion of such short fragments in degraded DNA samples has been identified as a problem since the beginning of fossil DNA studies, as mentioned in (Soltis and Soltis 1993; Zimmermann *et al* 2008). Since the DNA extracts in such samples are highly fragmented, it is advisable to select a small amplicon size for any research work on degraded DNA samples from taxidermic skin, bones, etc (Rodrı´guez *et al* 2004; Martı´n *et al* 2007). Shorter barcoding regions or mini barcodes are of great importance as

only a few options are available for rare and threatened faunal species in barcoding initiatives and other molecular studies required for phylogenetic and ecological studies and wildlife forensics.

Acknowledgments

The authors are grateful to the Director, Zoological Survey of India, Dr. K. Venkataraman for providing all facilities and encouragement. They are also thankful to the Officer-in-Charge, Northern Regional Centre, Zoological Survey of India for support and to the Mammals Section, Zoological Survey of India, Kolkata for providing taxidermic skin samples. The study was carried out as part of a project funded by the Department of Science & Technology, New Delhi, India.

References

1. Baker RJ (1994) Some thoughts on conservation, biodiversity, museums, molecular characters, systematics, and basic research. *J. Mamm***75**:277–287.
2. Weber DS, Stewart BS, Garza JC and Lehman N (2000) An empirical genetic assessment of the severity of the northern elephant seal population bottleneck. *CurrBiol***10**:1287–1290.
3. Houde P, Braun MJ (1988) Museum collections as a source of DNA for studies of avian phylogeny. *Auk***105**:773–776.
4. Cooper A (1994) DNA from museum specimens. In: Ancient DNA, edited by Herrmann B, Hummel S. New York: *Springer*. Pp. 149–165.
5. Cooper A (1997) Studies of avian ancient DNA: From Jurassic Park to modern island extinctions. In: Avian Molecular Evolution and Systematics, edited by Mindell DP. San Diego: Academic Press. Pp. 345–373.
6. Sheldon FH (2001) Molecular collections for basic research: Museums, methods, and morality. In: Proceedings of the International Conference of In Situ and Ex Situ Biodiversity Conservation in the New Millennium, edited by Yaacob Z, Moo-Tan S, Yorath S. Pp. 331–346.
7. Willerslev E, Cooper A (2005) Ancient DNA. *Proc R Soc*: B 272:3–16.
8. Wandeler P, Hoeck PEA, Keller LF (2007) Back to future: Museum specimens inpopulation genetics. *Trends EcolEvol***22**:634–642.
9. Kruckenhauser L, Haring E (2010) Advantages and limits of DNA analyses of specimens from scientific museum collections. In: Collections in Context, Proceedings of the 5th International Meeting of European Bird Curators, edited by Bauernfeind E, Gamauf A, Berg H-M. Wien. 225–235.
10. Payne RB, Sorenson MD (2002) Museum collections as sources of genetic data. *Bonner zoolBeitr***51**:97–104.
11. Shedlock AM, Haygood MG, Pietsch TW, Bentzin P (1997) Enhanced DNA extraction and PCR amplification of mitochondrial genes from formalin-fixed museum specimens. *Biotechniques***22**:394–396.
12. Yang, H, Golenberg EM, Shoshani J (1997) Proboscidean DNA from museum and fossil specimens: An assessment of ancient DNA extraction and amplification techniques. *Biochem Genet***35**:165–179.
13. Pichler FB, Dalebout ML, Baker CS (2001) Nondestructive DNA extraction from sperm whale teeth and scrimshaw. *MolEcol Notes***1**:106–109.
14. Junqueira ACM, Lessinger AC, Azeredo-Espin AML (2002) Methods for the recovery of mitochondrial DNA sequences from museum specimens of myiasis-causing flies. *Med Vet Entomol***16**:39–45.
15. Marte F, Péquignot A, von Endt DW (2006) Arsenic in taxidermy collections: history, detection, and management. Collection Forum 21:143–150.
16. Topfer T, Gamauf A, Haring E (2011) Utility of arsenic-treated bird skins for DNA extraction. *BMC Research Notes*4:197.
17. Barros NDM, Morgante JS (2007) A simple protocol for the extraction and sequence analysis of DNA from study skin of museum collections. *Genetics & Mol. Bio.* **30**,4:1181-1185.
18. Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, Wilson AC (1989) Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proc Natl Acad Sci USA***86**:6196–6200.
19. Ho¨ss M, Pa¨a¨bo S (1993) DNA extraction from Pleistocene bones by a silica based purification method. *Nucl Acids Res* **21**:3913–3914.
20. Ha¨nni C, Brousseau T, Laudet V, Stehelin D (1995) Isopropanol precipitation removes PCR inhibitors from ancient bone extracts. *Nucl Acids Res***23**:881–882.
21. Kalmar T, Bachrati CZ, Marcsik A, Rasko I (2000) A simple and efficient method for PCR amplifiable DNA extraction from ancient bones. *Nucl Acids Res***28**: E67.

22. Rohland N, Hofreiter M (2007) Ancient DNA extraction from bones and teeth. *Nature Protocols***2**:1756–1762.
23. Telle S, Thines M (2008) Amplification of cox2 (,620 bp) from 2 mg of up to 129 years old herbarium specimens, comparing 19 extraction methods and 15 polymerases. *PLOS ONE***3**: e3584.
24. Rohland N, Hofreiter M (2007) Comparison and optimization of ancient DNA extraction. *BioTechniques***42**:343–352.
25. Pa¨a¨bo S (1990) Amplifying ancient DNA. In PCR Protocols: A Guide to Methods and Applications, edited by Innis MA, Gelfand DH, Sninsky JJ, White TJ. New York: Academic Press. 159–166.
26. Oshida T, Lin LK, Masuda R, Yoshida MC (2000) Phylogenetic relationship among Asian species of Petaurista (Rodentia, Sciuridae), inferred from mitochondrial cytochrome b gene sequence. *Zoological Science* 17: 123-128.
27. Soltis PS, Soltis DE (1993) Ancient DNA: Prospects and limitations. *New Zealand J Bot***31**:203–209.
28. Zimmermann J, Hajibabaei M, Blackburn DC, Hanken J, Cantin E, et al. (2008) DNA damage in preserved specimens and tissue samples: A molecular assessment. *Frontiers of Zoology***5**:doi:10.1186/1742-9994-5-18.
29. Rodrı´guez MA, Garcı´a T, Gonza´ lez I, Asensio L, Herna´ndez PE, Martı´nR. (2004) PCR identification of beef, sheep, goat and pork in raw and heat-treated meat mixtures. *J Food Prot***67**:172–177.
30. Martı´n I, Garcı´a T, Fajardo v, Lo´ pez-Calleja I, Rojas M, Pavo´ n Ma, Herna´ ndezPE, Gonza´ lez I, Martı´n R. (2007) Technical note: Detection of chicken, turkey, duck, and goose tissues in feedstuffs using species-specific polymerase chain reaction.*J. Anim. Sci***85**:452–458.