academicJournals

Vol. 12(22), pp. 3399-3404, 29 May, 2013
DOI: 10.5897/AJB12.2759
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

An effective method for extraction and polymerase chain reaction (PCR) amplification of DNA from formalin preserved tissue samples of snow leopard

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Accepted 13 May, 2013

Formalin-preserved biological samples obtained from endangered species are valuable in assessing genetic diversity. To make use of snow leopard samples preserved in formalin over a period of two to seven years, we optimized the method of extracting DNA from these samples. We used (a) phenol chloroform : isoamyl alcohol, (b) the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany), (c) the Qiagen DNeasy Blood and Tissue Kit after treating the samples with NaOH for three days and (d) the Qiagen DNeasy Blood and Tissue Kit after treating the samples with phosphate buffered saline (PBS) for three days. The usefulness of the extracted DNA was assessed on the basis of mitochondrial (150 to 550 bp) and nuclear (95 to 229 bp) markers. There was no PCR amplification with the first two methods. The PCR amplification with the NaOH and PBS treatment had a success rate of 30 to 100% for both mitochondrial and nuclear markers. The PBS method is the best method for extraction of DNA from formalin-preserved samples of longer period (two to seven years) because of higher success rate in amplifying mitochondrial gene of ca. 550 bp (60%) than the NaOH method (28%). The overall amplification of microsatellite markers in such samples was also higher in samples treated with PBS (43 to 100%) than NaOH (0 to 100%). The PCR products obtained were confirmed through DNA sequencing to be of snow leopard origin. The optimized protocol will enable genetic studies to be conducted on tissue samples of other species that have been preserved in formalin. The protocol will be particularly useful for species that are elusive and from which it is difficult to collect fresh tissue samples.

Key words: Formalin, polymerase chain reaction (PCR), mtDNA, microsatellites, snow leopard.

INTRODUCTION

Obtaining samples of endangered wildlife species for genetic study is laborious and costly, and so it is mostly museum samples that are used for phylogenetic and taxonomic studies. Specimens in museum collections are often the last and only sources available for genetic analysis and molecular studies, such as when a population and species are on the verge of extinction (Schander and Halanych, 2003). This museum material is also used to compare present-day and historical genetic structure and to assess changes arising in genetic structure due to habitat changes (Larson et al., 2002).

Formalin is the most suitable and economical fluid for preserving soft tissues. Formalin-preserved specimens also require the least maintenance. As a result, formalin is the most commonly used fluid for preserving vertebrate specimens (Rodriguez et al., 2002; Bucklina and Allen, 2004). But extraction of DNA from museum samples preserved in formalin is difficult, and therefore such

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samples are of limited use for molecular studies. The difficulty in extracting DNA from formalin-preserved tissue samples arises from the various adverse effects, both direct and indirect, that formalin has on the structure of DNA. Formalin forms cross-links between the DNA and cellular protein, and DNA is degraded by unbuffered (pH not maintained) solutions of formalin as the pH values of such solutions can be very low (<1) when preserved for long duration. It undergoes irreversible denaturation, modification and fragmentation (Chaw et al., 1980; Paabo et al., 1989; Chang and Loew, 1994; Gilbert et al., 2007). Based on the fact that 4% formalin is slightly acidic (French and Edsall, 1945), buffered formalin solutions (pH 7) prepared by adding chemicals such as sodium dihydrogen phosphate have been used for preserving tissue samples, but when these are stored for long periods, they become very acidic (Means and Feeney, 1995). The pH value of a formalin solution may change from 5 (at the time of preservation) to 2 (after preservation for a long period), causing denaturation and fragmentation of DNA (Koshiba et al*.,* 1993; Kosel and Graeber, 1994).

Mitochondrial DNA and nuclear markers are used in phylogenetics (Moore, 1995), evolutionary studies (Avise, 2000) and genetic characterization at the spatial level (Anderson et al., 2010). Fresh samples of many mammalian species are not available for genetic studies due to declines in populations as a result of poaching and other factors. Thus museum specimens are valuable biological references for comparative genetic studies of species. The condition of a museum specimen and its quality (in terms of use for DNA analysis using mtDNA and nuclear markers) are important in molecular and phylogenetic studies and in conservation genetics. DNA extraction with the formalin-preserved samples and PCR amplification from both nuclear and mitochondrial genes has been attempted from different species (fishes, mammals, snakes, etc.), and the success rate has been between 12 and 74% for 100 to 400-bp for both markers (Rassman et al., 1991; Rodriguez et al., 2002; Gilbert et al., 2007; Friedman and Dessalle, 2008; Palero et al., 2010; Raja et al., 2011).

The snow leopard (*Uncia uncia*) is one of the most elusive large cats, and it is difficult to study. It belongs to the family Felidae, order Carnivora, and is categorized as Endangered under C1 by the International Union for Conservation of Nature (IUCN). It is found at high altitudes in mountain ranges in Central Asia, including the Tien, Kun Lun, Pamir, Hindu Kush, Karakoram and Himalayan ranges (Nowell and Jackson, 1996; Sunquist and Sunquist, 2002; McCarthy and Chapron, 2003). It is listed in Schedule I of the Wildlife (Protection) Act, 1972 of India. The snow leopard is restricted to the alpine and sub-alpine ecological zones, and the major threats it faces are related to degradation and fragmentation of its habitat, a lack of effective implementation and enforcement of laws and poaching of its natural prey for illegal trade across snow leopard range. In addition, snow leopards are also killed for their body parts and skin, which are used in various high-value products. There is local human-snow leopard conflict due to predation of livestock, and there is lack of trans-boundary cooperation and military activity in snow leopard habitats (McCarthy et al., 2003). Local populations have declined sharply over the past decade, and only 3500 to 7000 individuals are globally left in the wild. In India, only 200 to 600 snow leopards remain in the wild (McCarthy et al., 2003). Obtaining biological samples of this elusive, far-ranging species, living in inhospitable habitats, to undertake genetic studies is very difficult. Tissue samples of this species that have been collected by the Forest Departments of Himachal Pradesh and Uttarakhand from dead animals and have been preserved in formalin over a period of 2 to 7 years are available.

The objective of this study was to optimize a protocol for extraction of DNA from formalin-preserved tissue samples for longer period and determine amplification success of both mitochondrial and nuclear genes of higher amplicon sizes so as to use them in wildlife forensic studies and conservation genetics.

MATERIALS AND METHODS

DNA extraction

The most commonly used method for obtaining DNA from different biological samples involves treatment with an alkaline reagent (such as sodium hydroxide) and neutralization with a suitable buffer (Rudbeck and Dissing, 1998). Many studies have been carried out on pre-extraction treatment of formalin-preserved samples to increase the quality and yield of the DNA. Pre-treatment methods often used include: (1) treatment with a hot alkali and addition of NaOH (with a different pH value and at a different temperatures) to the digestion buffer (Shi et al., 2002, 2004), (2) adding glycene to the digestion buffer as a binding agent to release the formalin (Shedlock et al., 1997) and (3) critical point drying using graded ethanol washes (Fang et al., 2002). Further, NaOH and phosphate buffered saline (PBS) have been used as a washing buffer for removing formalin from preserved samples by enhancing the pH value prior to the extraction (Chase et al., 1998; Shi et al., 2002, 2004; Diaz-Viloria et al., 2005). Most of these studies have been limited to samples preserved for a short duration in formalin. Studies have indicated that the use of PBS and NaOH is relatively shorter and saves time. Therefore, we decided to use PBS and NaOH treatments in the present study.

We used a total of seven snow leopard tissue samples (n = 7) preserved in formalin for a period of two to seven years. These were provided by the Forest Departments of Himachal Pradesh and Uttarakhand. We used four methods to extract DNA from the samples, namely using (a) phenol chloroform : isoamyl alcohol (Sambrook et al., 1989), (b) the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany), (c) the Qiagen DNeasy Blood and Tissue Kit after treatment with NaOH for three days and (d) the Qiagen DNeasy Blood and Tissue Kit after treatment with PBS for three days.

The tissue samples were cut into small pieces in Petri dishes. Pieces (100 mg) were placed in 1.5-ml Eppendorf tubes without any pulverization. DNA was extracted from them using phenol chloroform: isoamyl alcohol (Sambrook et al., 1989) and the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the standard protocol and the manufacturer's instructions.

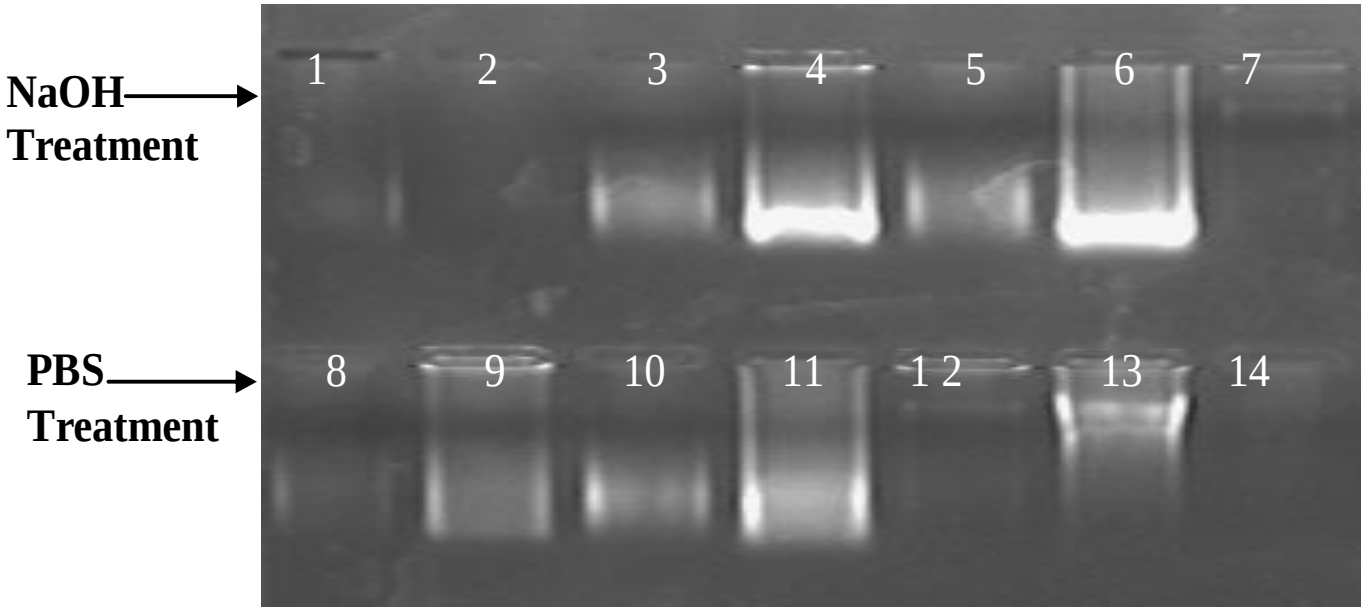

Figure 1. DNA electrophoresed on 0.8% agarose gel after DNA extraction. Lanes 1 to 7, NaOH-treated samples; lanes 8 to 14, PBS-treated samples.

We also washed two sets of seven samples each with NaOH and PBS separately before extracting DNA. We added 800 µl of NaOH and PBS, respectively to the two sets. We placed both the sets in a rotating chamber and removed the NaOH and PBS at intervals of 8 h for three days. When the tissue samples started dissolving in the NaOH and PBS, we stopped adding any further NaOH and PBS to the tubes. Then we removed the NaOH and PBS from the tubes. After three days, all the samples were centrifuged at 6000x *g* for 1 min. The supernatant of NaOH and PBS was discarded, and subsequently the DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany). The extracted DNA was checked on 0.8% agarose gel by loading 1 µl of loading dye (bromophenol blue) and 4 µl of DNA extract (Figure 1).

PCR amplification of mitochondrial DNA (mtDNA)

To assess the size of amplifiable DNA present in the formalin-preserved samples, we carried out polymerase chain reaction (PCR) amplification of the extracted DNA with universal primers of different sizes (151 to 550 bp) of mtDNA genes, viz. 12S rRNA (151 bp) (Rohland et al., 2004), 12S rRNA (384 bp) (Kocher et al., 1989) and 16S rRNA (550 bp) (Guha et al., 2006). The composition of the PCR master mix for 10 µl of reaction volume was 1× PCR buffer, 2 mM of $MgCl_2$, 200 µM of dNTP, 0.2 µM of each primer, 0.5 U Taq polymerase (MBI, Fermantas) and 40 ng of template DNA. The PCR thermal cycling included initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 45 s, with a final extension for 20 min at 72°C. Amplification was checked by loading 4 µl of the reaction mixture on a 2% (w/v) agarose gel. The confirmed PCR products were cycle sequenced and purified using the BigDye Terminator and clean-up method. These products were then subjected to DNA sequencing on an ABI 3130 Genetic Analyzer. The sequences obtained were examined using SEQUENCING ANALYSIS 5.2. (Applied Biosystems) and validated at NCBI BLAST (http://blast.ncbi.nlm.nih.gov/ blast. cgi). The NCBI validation confirmed that the amplified amplicons were only from snow leopards.

PCR amplification of microsatellite loci

Multilocus genotyping (species-specific and heterologous loci) is a powerful tool in conservation genetics (Janecka et al., 2008; Waits et al., 2007). Therefore, we also assessed the success rate due to different treatments of microsatellite loci specific to the snow leopard (n = 3), heterologous loci specific to the tiger (n = 2) and the domestic cat (n = 2). PCR amplification of seven fluorescent labeled polymorphic microsatellite loci of different sizes (90 to 264 bp) was carried out in singleplex PCR: PUN82 (110 to 115 bp) and PUN124 (90 to 100 bp) of the snow leopard (Janecka et al., 2008); E5 (174 to 190 bp), 4A (105 to 297 bp) and A2 (182 to 196 bp) of the tiger (Sharma et al., 2008); and FCA724 (202 to 218 bp) and FCA723 (194 to 264 bp) of the domestic cat (Menotti-Raymond et al., 1999). The composition of 10 µl of the PCR master mix of the reaction volume was 1× PCR buffer, 2 mM of $MgCl_2$, 200 µM of dNTP, 0.4 µM of each primer, 0.5 U Taq polymerase (MBI, Fermantas) and 40 ng of template DNA. The PCR thermal cycling included initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 45 s, annealing at 51 to 53°C for 45 s and extension at 72°C for 45 s, with a final extension for 20 min at 72°C. The amplification was checked by loading 4 µl of the reaction mixture on a 2.0% (w/v) agarose gel using a 100 to 1000 bp ladder, and some of the allele sizes were confirmed using GeneMapper 3.7.

RESULTS AND DISCUSSION

We excluded from our analysis the results of the first two methods which were without any treatment, namely the phenol chloroform : isoamyl alcohol method (Sambrook et al., 1989) and one with the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) because the DNA was invisible on 0.8% agarose gel. Extraction of DNA was successfully accomplished after treatment with PBS and NaOH as DNA visible on agarose gel (Figure 1).

Table 1. Success rate (percentage) of PCR amplification of mtDNA genes using DNA extracted from formalin-preserved snow leopard tissue samples (n = 7) after NaOH and PBS treatment.

Gene	Size (bp)	Treatment	
		NaOH	PBS
12S rRNA	151	100	100
12S rRNA	384	100	86
16S rRNA	550	28	60

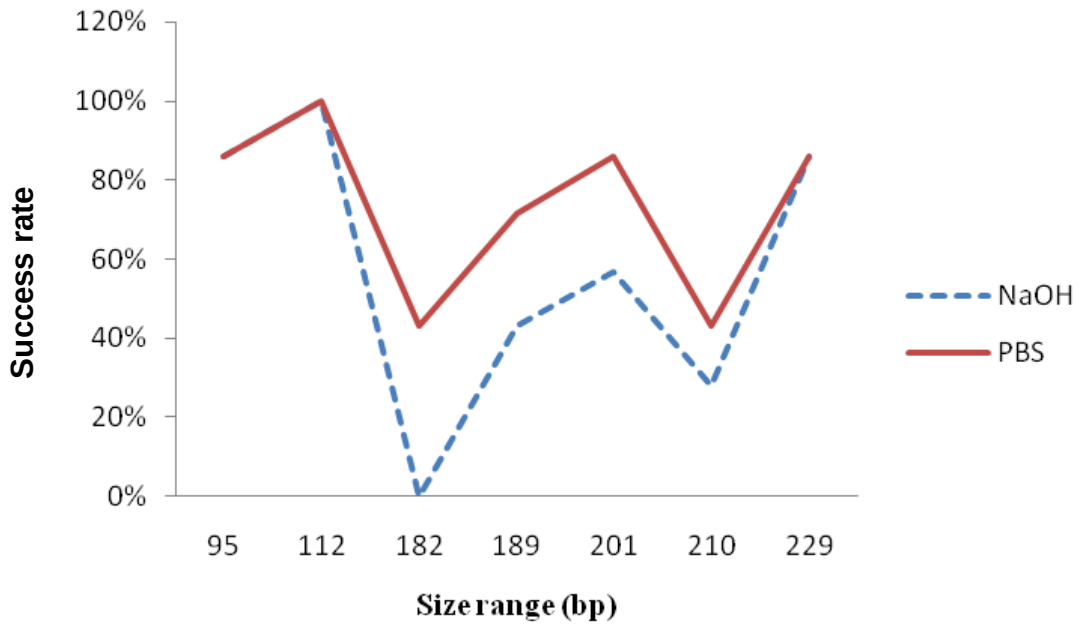

Figure 2. Success rate of PCR amplification of microsatellite loci using DNA extracted from formalin-preserved snow leopard tissue samples (n = 7) after NaOH and PBS treatment.

The success in PCR amplification of the 12S rRNA primer (<384 bp) was between 80 and 100% whereas the success was 100% with the 12S rRNA primer of 151 bp. With 550 bp 16S rRNA, the success was 30 and 60% in samples treated with NaOH and PBS, respectively (Table 1).

All the microsatellite loci (n = 7) were amplified in the samples, but the success varied with treatment and locus (Figure 2). There was no relationship between the success and the size of a microsatellite locus. However, there was better success in PCR amplification (43 to 100%) of all seven loci with samples treated with PBS, whereas the success was only 0 to 100% in samples treated with NaOH. We had 100% success in PCR amplification with PUN82 (110 to 115 bp) in both samples treated with NaOH and those treated with PBS. The success was >60% with five loci out of seven loci with samples treated with PBS, as compared the samples treated with NaOH (>43%). Thus, we concluded that the PBS pre-treatment method is better than the method involving pre-treatment with NaOH for amplification of nuclear markers.

Fixing biological samples using formalin degrades the DNA. Formalin is a potent agent, causing cross-linking of DNA with DNA, DNA with proteins, and proteins with proteins. Degradation occurs at room temperature with formalin fixation because of the presence of formic acid, coupled with low pH values and a low salt concentration (Koshiba et al., 1993; Shi et al., 2004). Because of the cross-linking and degradation of DNA, very little free double-strand DNA is released during extraction (Trifonov et al., 1967; McGhee and Von Hippel, 1975a, b, 1976a, b). Such cross-linking can be partially broken, and only limited success is possible in PCR amplification and electrophoresis. At a neutral pH value 7, formalin reacts with three bases in the DNA fragment, namely cytosine, guanine and adenine, which leads to the formation of reactive formaldehyde compounds via the methylene group. This inhibits primer annealing and renaturation and suppresses the replication process during the PCR reaction (McGhee and Von Hippel, 1975a, b; Neubauer et al., 1992; Karlsen et al., 1994). It was reported that 2.5% DNA-protein cross-linking remained in the DNA which made the polymerase enzyme to fail during the

PCR amplification of markers of size greater than 200 bp (Karlsen et al., 1994). Several studies have been conducted on extraction of DNA from formalin-preserved samples (Shiozawa et al., 1992; Cano and Poinar, 1993; Shedlock et al., 1997; Chase et al., 1998), but the success has been highly inconsistent.

Chakraborty et al. (2006) reported that they had no success with amplicons of the target gene (400 bp) of 12S and 16S rRNA with PCR amplification using published protocols (Walsh et al., 1991; Asahida et al., 1996; Shedlock et al., 1997; Wirgin et al., 1997; Qiagen, Germany) for the samples that had been stored for long periods (3 to 4 years) in formalin. They concluded that the DNA structure is less affected by covalent cross-linking, irreversible denudation, modification, etc. when a specimen is stored for a short period (7 days) in formalin, and that it is hence easy to extract DNA from such samples and amplify mtDNA markers of size up to 400 to 550 bp (Chakraborty et al., 2006). Koshiba et al. (1993) suggested that amplification of PCR products of up to 600 bp is possible with specimens fixed in buffered formalin and stored at 4°C for short periods (7 days). Likewise, a reduced exposure of samples to formalin and fixation at temperatures close to about 4°C greatly reduces the degradation of DNA, and extraction from these samples is easy (Koshiba et al., 1993). Palero et al. (2010) reported a low success rate with formalin-preserved fish samples with an amplicon size of 250 to 300 bp. The literature indicates that it is possible to get PCR amplicons of size 100 to 200 bp from samples preserved for longer periods in formalin (3 to 25 years). The applicability of such data is limited to phylogenetic and genetic studies of closely related species (Bucklina and Allena, 2004; Chakraborty et al., 2006) due to short DNA sequences.

Our study reveals that the DNA extraction from samples that have been preserved in formalin for long periods, that is, two to seven years, is possible with NaOH and PBS treatments with a success of >80% for 384-bp 12S rRNA. It is possible to have a success of >60% with 550-bp 16S rRNA with samples treated with PBS. The success with microsatellites was highly variable, and there was no consistency across microsatellite loci of different sizes (Figure 2). Therefore we suggest that more different loci of low to moderate amplicon size (<230 bp) be selected and screened for the applicability of the method to snow leopard samples.

Our optimized method has great potential for use with historical formalin-preserved samples in phylogenetic studies and conservation genetics. Besides, our method is of significance for wildlife forensics as samples seized by enforcement agencies in wildlife offence cases are often stored in formalin and are crucial for implementation of national and international acts and treaties.

ACKNOWLEDGEMENTS

We extend our thanks to the Director, Dean and Research Coordinator, Wildlife Institute of India, Dehradun, for their consistent support and for their encouragement during this work. We would like to express our gratitude to our laboratory colleagues for sharing their personal experiences with us and for valuable discussion.

REFERENCES

Anderson CD, Epperson BK, Fortin MJ, Holderegger R, James PMA, Rosenberg MS, Kim T, Scribner KT, Spear S (2010). Considering spatial and temporal scale in landscape-genetic studies of gene flow. Mol. Ecol. 19: 3565–3575.

Asahida T, Kobayashi T, Saito K, Nakayama I (1996). Tissue preservation and total DNA extraction from fish stored at ambient temperature using buffers containing high concentration of urea. Fish. Sci. 62: 727–730.

Avise JC (2000). Phylogeography: The History and Formation of Species. Harvard University Press, Cambridge.

Bucklina A, Allena LD (2004). mtDNA sequencing from zooplankton after long-term preservation in buffered formalin. Mol. Phyl. Evol. 30: 879–882.

Cano RJ, Poinar H (1993). Rapid isolation of DNA from fossil and museum specimens suitable for PCR. Biol. Technol. 15: 432–435.

Chakraborty A, Sakai M, Iwatsuki Y (2006). Museum fish specimens and molecular taxonomy: A comparative study on DNA extraction protocols and preservation techniques. J. Appl. Ichthyol. 22: 160–166.

Chang YT, Loew GH (1994). Reaction mechanisms of formaldehyde with endocyclic imino groups of nucleic acid bases. J. Am. Chem. Soc. 116: 3548–3555.

Chase MR, Etter RJ, Rex MA, Quattro JM (1998). Extraction and amplification of mitochondrial DNA from formalin-fixed deep-sea molluscs. Biol. Technol. 24: 243–247.

Chaw YM, Crane LE, Lange P, Shapiro R (1980). Isolation and identifications of cross-linked from formaldehyde treated nucleic acid. Biochemistry 19: 5525–5531.

Diaz-Viloria N, Velasco N and Perez-Enriquez R (2005). Inhibition of DNA amplification in marine fish larvae preserved in formalin. J. Plank. Res. 27(8): 787–792.

Fang SG, Wan QH, Fujihara N (2002). Formalin removal from archival tissues by Critical Point Drying. Biotechnology 33: 604–611.

French D, Edsall JT (1945).The reaction of formaldehyde with amino acid and proteins. in: Anson ML and Edsall JT (eds.), Adv. Prot. Chem. Academic Press, New York. 2: 1-135.

Friedman M, Desalle R (2008). Mitochondrial DNA extraction and sequencing of formalin-fixed archival snake tissue. Mitoch. DNA 19(5): 433–437.

Gilbert MT, Haselkorn T, Bunce M, Sanchez JJ, Lucas SB, Jewell LD, Van Marck E, Worobey M (2007). The isolation of nucleic acids from fixed, paraffin-embedded tissues: Which methods are useful when? PloS One 2(6): 1–12.

Guha S, Goyal SP, Kashyap VK (2006). Genomic variation in the mitochondrially encoded cytochrome b (MT-CYB) and 16S rRNA (MT-RNR2) genes: Characterization of eight endangered pecoran species. Ani. Gene. 37: 262–265.

Janecka JE, Jackson R, Yuquang Z, Diqiang L, Munkhtsog B, Buckley-Beason V, Murphy WJ (2008). Population monitoring of snow leopards using noninvasive collection of scat samples: A pilot study. Ani. Cons. 11(5): 401-411.

Karlsen F, Kalantari M, Chetemerere M, Johansson B, Hagmar B (1994). Modification of human and viral deoxyribonucleic acid by formaldehyde fixation. Lab. Investi. 71: 504–611.

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.

Kosel S, Graeber MB (1994). Use of neuropathological tissue for molecular genetic studies: Parameters affecting DNA extraction and polymerase chain reaction. Acta. Neuropathol. 88: 19–25.

Koshiba M, Ogawa K, Hamazaki S (1993). The effect of formalin fixation on DNA and the extraction of high-molecular-weight DNA from fixed and embedded tissues. Pathol. Res. Pract. 89: 66–72.

Larson SE, Jameson R, Etnier M, Fleming M, Bentzen P (2002). Loss of genetic diversity in sea otters (*Enhydra lutris*) associated with the fur trade of the 18th and 19th centuries. Mol. Ecol. 11: 1899–1903.
McCarthy TM, Allen P M, Fox J, Chapron G, Jackson R M, Mishra C, Theile S (2003). Snow leopard survival strategy. ISLT, Seattle.
McCarthy TM, Chapron G (2003). Snow Leopard Survival Strategy. International Snow Leopard Trust and Snow Leopard Network, Seattle, USA.
McGhee JD, Von Hippel PH (1975a). Formaldehyde as a probe of DNA structure. 1. Reaction with exocyclic amino group of DNA bases. Biochemistry 14: 1281–1296.
McGhee JD, Von Hippel PH (1975b). Formaldehyde as a probe of DNA structure 2. Reaction with endocyclic amino group of DNA bases. Biochem. 14: 1297–1303.
McGhee JD, Von Hippel PH (1976a). Formaldehyde as a probe of DNA structure. 3. Mechanism of initial reaction of formaldehyde with DNA. Biochemistry16: 3267–3276.
McGhee JD, Von Hippel PH (1976b). Formaldehyde as a probe of DNA structure 4. Equilibrium denaturation of DNA and synthetic polynucleotides. Biochemistry 16: 3276–3293.
Means GE, Feeney RE (1995). Reductive alkylation of proteins. Anal. Biochem. 224: 1–16.
Menotti-Raymond M, David VA, Lyons LA, Schaffer AA, Tomlin JF, Hutton MK, O'Brien SJ (1999). A genetic linkage map of microsatellites in the domestic cat (*Felis catus*). Genomics 57: 9–23.
Moore WS (1995). Inferring phylogenies from mtDNA variation: Mitochondrial-gene trees versus nuclear-gene trees. Evolution 49: 718–726.
Neubauer A, Neubauer B, He M, Effect P, Iglehart D, Frye RA, Liu E (1992). Analysis of gene amplification in archival tissue by differential PCR. Oncogene 7: 1019-1025.
Nowell K, Jackson P (1996). Wild cats: Status, survey and conservation action plan. Gland, Switzerland: IUCN.
Paabo S, Higuchi RG, Wilson AT (1989). Ancient DNA and the polymerase chain reaction. J. Biol. Chem. 264: 9709–9712.
Palero F, Hall S, Clark PF, Johnston D, Dodds JM, Thatje S (2010). DNA extraction from formalin-fixed tissue: New light from the deep sea. Sci. Marine 74(3): 465–470. Barcelona (Spain) ISSN: 0214-8358doi: 10.3989/scimar.2010.74n3465
Raja M, Muralidharan M, Arunchalam M (2011). Comparative analysis of DNA extracted from fish species preserved in formalin in two different periods. Turk. J. Biol. 35 Tubitak, doi:10.3906/biy-0908-11
Rassman K, Schlotterer C, Tautz D (1991). Isolation of simple sequence loci for use in PCR-based DNA fingerprinting. Electrophoresis 12: 113-118.
Rodriguez R, Bastida R, Olsson P (2002). DNA extraction from formalin fixed franciscana tissue. LAJAM. 1: 123–128.
Rohland N, Siedel H, Hofreiter M (2004). Nondestructive DNA extraction method for mitochondrial DNA analyses of museum specimens. Biol. Technol. 36: 814–821.
Rudbeck L, Dissing J (1998). Rapid, simple alkaline extraction of human genomic DNA from whole blood, buccal epithelial cells, semen and forensic stains for PCR. Biol. Technol. 25: 588-592.
Sambrook J, Fritsch EF, Manlatis T (1989). Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press.
Schander C, Halanych KM (2003). DNA, PCR and formalinized animal tissue: A short review and protocols. Org. Divers. Evolution 3: 195–205.
Sharma S, Stuckas H, Moll K, Khan I, Bhaskar R, Goyal SP, Tiedemann R (2008). Fourteen new dinucleotide and tetranucleotide microsatellite loci for the critically endangered Indian tiger (*Panthera tigris tigris*). Mol. Ecol. 8: 1480–1482.
Shedlock AM, Haygood MG, Pietsch TW, Bentzen P (1997). Enhanced DNA extraction and PCR amplification of mitochondrial genes from formalin-fixed museum specimens. Biol. Technol. 22: 394–400.
Shi SR, Cote RJ, Wu L, Liu C, Datar R (2002). DNA extraction from archival formalin-fixed, paraffin-embedded tissue sections based on the antigen retrieval principle: Heating under the influence of pH. J. Histochem. Cytochem. 50: 1005–1011.
Shi SR, Liu DC, Wu L, Zhang Z, Cote R, Taylor C (2004). DNA extraction from archival formalin-fixed, paraffin-embedded tissues: Heat-induced retrieval in alkaline solution. Histochem. Cell Biol. 122: 211–218.
Shiozawa DK, Kudo J, Evans RP, Woodward SR, Williams RN (1992). DNA extraction from preserved trout tissues. Great. Basin. Nat. 52: 29–34.
Sunquist ME and Sunquist F (2002). Wild Cats of the World. Chicago, IL: University of Chicago Press.
Trifonov EN, Lazurikin YS, Frank-Kamenstsky MD (1967). Formaldehyde- induced uncoiling of DNA. Mol. Biol. 1: 142–151.
Waits LP, Buckley-Beason VA, Johnson WE, Onorato D, McCarthy T (2007). A select panel of polymorphic microsatellite loci for individual identification of snow leopards (*Panthera uncia*). Mol. Ecol. Not. 7: 311–314.
Walsh PS, Metzger DA, Higuchi R (1991). Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biol. Technol. 10: 506–513.
Wirgin I, Maceda L, Stabile J, Mesing C (1997). An evaluation of introgression of Atlantic coast striped bass mitochondrial DNA in a Gulf of Mexico population using formalin-preserved museum collections. Mol. Ecol. 6: 907–916.