The Journal of Animal & Plant Sciences, 25(4): 2015, Page: 1196-1199
ISSN: 1018-7081

Short Communication

EVALUATION OF DNA EXTRACTION METHOD FROM FORMALIN PRESERVED SKIN SAMPLES OF *PANTHERA PARDUS* FOR MOLECULAR GENETIC ASSESSMENT

S. S. Bibi[1], A. Rehman[2*], R. A. Minhas[1] and S. Janjua[2*]

[1]Department of Zoology, University of Azad Jammu and Kashmir, Muzaffarabad; [2]Bioresource Research Centre (BRC), 34-Bazar Road, G-6/4 Islamabad
[*]Corresponding author's email: safiajanjua@hotmail.com

ABSTRACT

Five different protocols, *viz.* Andleeb *et al.* (2012), Shrivastava *et al*. (2012), Phenol chloroform DNA extraction method, Roboscreen Kit method and Phenol chloroform triton method, were tested for DNA extraction from skin samples of Leopard (*Panthera pardus)* preserved in formalin over a period of two to five months. Among these procedures, the Phenol chloroform triton method was proved to be the most efficient protocol with 83% extraction rate and revealed 100% amplification of FCA026 and FCA043 loci. Keeping in views the extraction and amplification efficiency, Phenol chloroform triton method is recommended for molecular studies of formalin preserved skin samples of *Panthera pardus*.

Key words: Skin Tissue Samples, DNA Extraction, Polymerase Chain Reaction

INTRODUCTION

Samples acquisition of endangered wildlife species for genetic studies is remained very laborious and costly. Hence, museum preserved samples are usually used for phylogenetic and taxonomic studies (Schander and Halanych, 2003). Formalin is the most commonly used fluid for preserving vertebrate specimens (Rodriguez *et al.,* 2002; Bucklina and Allen, 2004). However, extraction of DNA from museum samples preserved in formalin is usually difficult, and therefore, such samples are of limited use for molecular studies. The difficulty in extracting DNA from formalin-preserved tissue samples arises from the various adverse effects as 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 low (3 to 4.6) 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).

The common leopard (*Panthera pardus*) is one of the most elusive large cats that belong to Felidae family (Nowell and Jackson, 1996). It is considered as Near Threatened on the IUCN Red List because its population is declining in large parts of its range due to habitat loss, fragmentation and hunting for trade and pest control. The status assessment of mammals categorized the common leopard as a Critically Endangered species in Pakistan (Sheikh and Molur, 2005). Therefore, knowledge of phylogentic history and genetic variation of common leopard in Pakistan is of fundamental importance for its conservation. Keeping in view the importance of the species, we assessed different available protocols for their efficiency in DNA extraction and amplification from its formalin preserved 2-5 months skin samples of dead animals collected from different localities of Azad Jammu and Kashmir (AJK).

MATERIALS AND METHODS

Sampling area: For DNA extraction, tissue samples (skin/hair; n=18) were acquired from the AJK Wildlife Department which were collected from the leopards (*Panthera pardus*) killed by local hunters and farmers from different localities of AJK. These samples were preserved in different chemicals such as formalin and different salts. All samples were properly labeled and stored at -20°C before use in further processing. The collected skin samples were carried to Molecular Ecology Laboratory of Bioresource Research Centre, Islamabad.

DNA extraction: We used five methods to extract DNA from the samples; Modified Protocol for genomic DNA extraction by Andleeb *et al*. (2012), Phenol-Chloroform extraction (Sambrook *et al.,* 1989), Phenol-chloroform-Triton protocol, Extraction of genomic DNA from formalin fixed tissues by Shrivastava *et al*. (2012) and Roboscreen Kit. The tissue samples were cut into small pieces in petri dishes. Pieces (100 mg) were placed in 1.5ml centrifuge tubes. Beside the above already established protocols, we also modified phenol chloroform triton method to extract the DNA.

To modify phenol chloroform triton method, we washed these samples with dH_2O and TE buffer separately before extracting DNA. Then 1ml of dH_2O was added in samples and was centrifuged at 13000 rpm for 10 minutes and pellet was collected. Then added

100ml of TE buffer and samples were centrifuged at 13000rpm for 10 min and discarded supernatant. Then the DNA was extracted using the Phenol-chloroform-Triton method; 300 µl lysis solutions (200mM NaCl, 10mM Tris-Hcl, pH 8.0, 10mM EDTA, pH 8.0 and 0.1% Triton-100) was added along with about equal amount of 1M DTT (40µl) and 40µl Proteinase K (2 mg / ml). The lysate was then extracted by similar procedure in phenol chloroform and DNA precipitated with equal volume of isopropanol and 3M of sodium acetate. The DNA was washed with 70% ethanol and re-suspended in TE buffer (10 mM Tris HCl, pH 8.0, and 1 mMEDTA) and stored at -20°C until further use. The extracted DNA was checked on 1% agarose gel and quality and quantity of extracted DNA was assessed by spectrophotometric method.

PCR amplification: To assess the quality of amplifiable DNA present in the sample preserved in formalin and different chemical, we carried out polymerase chain reaction (PCR) amplification of the extracted DNA in a DNA Thermal Cycler (Thermo scientific PCR SPRINT THERMAL CYCLER). Two polymorphic microsatellites loci were amplified by using primers (selected from originally designed microsatellite for the domestic cat, *Felis catus* (Menotti-Raymond *et al*., 1999). Annealing temperature of the primers were first estimated by using the equation of Tm=4(G+C) +2(A+T) suggested by Fermentas manufacturer. PCR chemicals used in this study were obtained from Fermentas (FermentasUAB Lithuania).

PCR optimization assays: For optimization the concentrations of PCR buffer and $MgCl_2$ were kept constant. The first parameter assayed for the optimization stages was the annealing temperature of primer. PCR was performed using the standard concentrations. The best annealing temperature was then used for PCR amplification of other samples in study. Then the primer and dNTP concentrations used in PCR were optimized.

RESULTS AND DISCUSSION

DNA Extraction: During present study, the extraction of DNA was more successfully accomplished with phenol chloroform-triton modified protocol (83.3% success rate).

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 Glycine 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. All these methods are time consuming so we modified the phenol chloroform method by carrying a washing (with TE buffer and dH_2O) as first step that is relatively shorter.

Extraction of genomic DNA from formalin fixed tissues by Shrivastava *et al*. (2012) and Andleeb *et al.* (2012) method was not sufficient for further PCR amplification. While the success rate of Phenol chloroform method was 33%. It was found that among all these methods, the phenol chloroform modified method was the most efficient and successful protocol for DNA extraction from formalin preserved skin samples of common leopard with 83% success rate (Table 1).

The maximum quantity of DNA (220.11 ± 15.09 ng/µl) was obtained by phenol-chloroform triton modified method and minimum quantity was found by phenol-chloroform methods (199.13 ± 11.09 ng/µl). The quality of extracted DNA was the best for DNA obtained by phenol chloroform triton modified method (1.92 ± 0.10), followed by phenol chloroform method (1.72 ± 0.07) (Table 2).

PCR optimization

Annealing temperature: The best primer annealing temperature (Ta) in the study was specific for each primer and the fragment gene length was between 90-152 base pair. Initial and optimized Ta is given in Table 3.

Primer analysis and final concentration: The primer used in this study was selected from originally designed microsatellite for the domestic cat, *Felis catus* (Menotti-Raymond *et al*., 1999). Uphyrkina *et al.* (2001) also used these primers for leopard's phylogenetics study. The best possible DNA band with the least or no primer dimers indicated the ideal primer concentration.

Thermo profile modification of PCR cycle duration: The initial PCR profile suggested by the manufacturer with the shortest possible cycle duration was set as the starter for gene amplification. The initial thermal cycling step consisted of initial denaturation at 94°C for 5 min 35 cycles (denaturation at 94°Cfor 45s, primer annealing at different temperature for 60s and primer extension at 72°C for 1 min) followed by final extension single stage at 72°C for 1 min. Cycle duration assays were conducted to investigate the effective cycle time for leopard. DNA amplification and the implication of increasing cycle duration to PCR products. In this study, two duration assays with parallel duration time increment between denaturation and primer annealing stage were conducted by increasing 15s (30s, and 45s), and the best duration was 45s for denaturation and 60s for primer annealing. The results were indicated by bands on agarose gel.

Table 1. Comparison of DNA extraction and PCR amplification success rates of mtDNA genes from skin samples of *P. pardus* using different protocols.

Methods	Total Samples (#)	Successful Extraction (#)	% Success	Successful amplification (#)	% PCR Success rate
Modified Protocol for genomic DNA Extraction by Andleeb *et al*. (2012)	18	2	9%	0	0%
Phenol-chloroform extraction (Sambrook *et al*., 1989)	18	6	33%	3	50%
Phenol-chloroform-Triton (modified protocol)	18	15	83.3%	15	100%

Table 2. Quantity and quality of DNA (mean ± S.E) extracted with different extraction methods from skins of *P. pardus* from different localities of AJK (n=18 for two methods).

DNA Extraction methods	Quantity (ng/µl)	Quality (A260/280)
Phenol - chloroform extraction (Sambrook *et al.,* 1989)	199.13 ± 11.09	1.76 ± 0.07
Phenol-chloroform-Triton (modified protocol)	220.11 ± 15.09	1.92 ± 0.10

Table 3: SSR Markers used to assess the genetic diversity in *P. pardus*

Primers		Primer Sequence (5´–3´)	Initial Ta	Ta (ºC)	Amplicon (bp)	References
FCA090	Forward	ATCAAAAGTCTTGAAGAGC	55	45	90-111	Menotti-Raymond *et al*., 1999 Uphyrkina *et al.,* 2001
	Reverse	TGTTAGCTCATGTTCATGTGTCC				
FCA043	Forward	GAGCCACCCTAGCACATATACC	58	52	111-115	Menotti-Raymond *et al*., 1999 Uphyrkina *et al.,* 2001
	Reverse	AGACGGGATTGCATGAAAA				
FCA026	Forward	GGAGCCCTTAGAGTCATGCA	56	50	150	Menotti-Raymond *et al*., 1999 Uphyrkina *et al.,* 2001
	Reverse	TGTACACGCACCAAAAACAA				
FCA075	Forward	ATGCTAATCAGTGGCATTTGG	54	49	112-146	Menotti-Raymond *et al*., 1999 Uphyrkina *et al.,* 2001
	Reverse	GAACAAAAATTCCAGACGTGC				

Conclusion: The study reveals that the DNA extraction from samples that have been preserved in formalin and other chemicals for long periods (two to five months) is possible by using phenol chloroform triton modified protocol with a success of 83%. For further validation of these results PCR was carried out using these extracted DNA samples. Our optimized method had great potential for use with formalin-preserved samples in phylogenetic studies and conservation genetics.

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