

Molecular Species Identification in Processed Animal Hides for Biodiversity Protection

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Abstract—Trade in endangered species causes a significant loss of biodiversity. Each year, millions of endangered animals are unlawfully killed or captured for trophy hunting, collections of private zoos, decorative objects, traditional medicine, or human consumption. Many species identification methods in processed organic products have been developed (morphological studies, Protein analysis, chemical analysis, etc.) but these methods are now outdated mainly because they are not very effective for the analysis of processed products. Thanks to derived methods of palaeogenetics, molecular identification methods have recently been developed. At an applied perspective, palaeogenetics has gained significant advances in the field of Criminalistics and Forensic science, as well as frauds detection and food quality control. Processed products such as leather, fur and all manufactured products made from animal hides are currently very reluctant to obtain reliable results in molecular traceability. However, the molecular identification of species origin of leather would be particularly important, either concerning the commercial aspect or more fundamental aspects. This study develops a methodology of extraction and amplification of DNA from leather, one of the most refractory to molecular analysis, because of the treatments used in their manufacture..

Keywords— DNA from leather, Species identification, Biodiversity protection

I. INTRODUCTION

THE illegal trade of endangered species causes a severe depletion of biodiversity [1] and the luxury trade of leather has pushed many species toward extinction (endangeredspecieshandbook.org) [2]. Endangered animals are often killed for meat, ornamental objects (e.g. elephant ivory [3] and traditional medicine (e.g. tiger [4, 5]). Thus, DNA technology, combined with the use of genetic barcode [6, 7], provides a sensitive, efficient and powerful way for species authentication using short DNA sequences [8]. Although obtaining reliable molecular traceability is possible from a large range of animal tissues, DNA profiling of leather is still very complex and difficult. Indeed, manufacturing treatments such as tanning and coloring can highly alter and

degrade DNA molecules [9, 10]. Moreover, another major problem is the co-extraction of inhibitors during DNA extraction process [10] that inhibits PCR amplifications. While the genetic determination of species origin of these specific samples has important applications for industrial and fundamental research goals [11, 12], very few studies have been developed. Basing on an improved DNA extraction method, as well as a couple of highly specific primers, we show in this study an accurate assay to identify the species origin in a leather of Chamois without any further dilution and/or purification of the extract..

II. MATERIALS AND METHODS

Degraded DNA techniques were used to retrieve DNA preserved in the leather. To avoid contamination, pre-amplification procedures (sampling, DNA extraction, PCR mix set-up) were carried out in the DNA Free platform, Palgene in Lyon, France, with drastic decontamination and wash procedures (respectively UV illumination and cleaning with Actril decontaminant solution). Moreover, manipulators wore protections such as full bodysuits, facemasks, and disposable gloves.

A. Sample

A piece of Chamois leather was provided by Hermès. This leather sample with one mm thickness was fully colored and treated with Alum tanning.

B. DNA extraction from leathers: *GuSCN/Silica DNA extraction*

Leather sample was manually reduced to tiny fibbers using sterile tweezers and blades. Five ml of extraction buffer containing 0,5M EDTA (pH 8), 1% N-Laurylsarcosine, 0,5mg/ml Proteinase-K were added to 100-500 mg of reduced sample and incubated 16-48 hr at 37°C under rotation. The remaining non-digested sample was pelleted by centrifugation. Then, extraction procedure was conducted on supernatant. A blank and different species samples (unpublished data) were co-extracted with samples during each extraction session to trace potential cross-contamination between samples and carrier effect [13, 14]. We applied the *GuSCN/Silica DNA extraction* already described in the literature [15] with modification of the binding and washing

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buffers composition as follows : Binding buffer (5M GuSCN, 50mM Tris, 25mM NaCl, 20 mM EDTA, Triton X-100 (1,3%), pH 8) and Washing buffer (70% ethanol). Elution was performed with 100 - 300 μ l of the Elution Buffer EB (Qiagen®).

C. Primers design and test on fresh DNA extracts

Primers were designed to amplify specifically short fragment of the mitochondrial gene 12sRNA (Table 1). All sequences of considered species were downloaded from the NCBI Genbank and aligned using Seaview [16]. Human sequences were aligned too, because one of the main possible contaminations can be the human DNA. Specific forward and reverse primers were purchased from Eurogentec®. To ensure highly specific amplifications, we tested the primers on fresh DNA extracts. Fresh samples have been extracted in a separate laboratory. Amplifications of primers tests have been cloned, sequenced and analyzed using BLASTn system (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Results have proven that the specific Chamois primers amplify only the DNA of target species and never amplify DNA of other species candidates in leather manufacture or human DNA.

TABLE I

| Primer ID | Sequence | Taret |
|-------------|------------------------------|------------------|
| Rup-12S-For | 5' CCCTCCTCAAGYRAATACAGGA 3' | 12s RNA |
| Rup-12S-Rev | 5' TGTTACGACTTGCTCTCTCTTG 3' | mtDNA Chamois |

Sequences of the forward end reverse chamois specific primers. The target is 76 bp in the 12sRNA gene within the mitochondrial DNA

D. PCR conditions

Optimal PCR conditions (and especially, MgCl₂ concentration and hybridization temperature) of primers were determined using fresh DNA. PCR reaction was carried out in a total volume of 25 μ l. PCR conditions were as follows: 2,5 units of Taq Gold polymerase (Perkin-Elmer®), 2,5 mM MgCl₂, 1 mg/ml CSA, 250 μ M of each dNTP (Sigma-Aldrich), and 0,5 μ M of each primer (Eurogentec®). Touchdown PCR approach was conducted: a first activation step (94°C - 10 min) was followed by 20 cycles (94°C - 30s, 60 to 50°C - 30s (decrease of 1°C/cycle) and 72°C - 30s). Then, 40 cycles were conducted (94°C - 30s, 50°C - 30s and 72°C - 30s). To finish, a final extension step at 72°C (10 min) was performed. One to five microliters of DNA extract was added. Three independent blanks were carried out for each set of PCR experiment as reported in Merheb *et al.* 2014 [8]. PCR products were loaded on 2 % agarose gel electrophoresis.

E. Sequencing

PCR products were further deep sequenced after cloning (Topo® TA cloning Kit; Invitrogen®). In the latter case, positive colonies for insertion were screened by PCR (35-45

cycles of denaturation: 94°C, 30 sec; annealing: 55°C, 30 sec; and elongation: 72°C, 45 sec) into a 22 μ l reaction mix consisting in 1 μ M of universal M13 primers (Forward: 5'-GTTTTCCCAGTCACGACGTTG; Reverse: 5'-TTTCACACAGGAAACAGCTAT) and 1X PCR supermix (Invitrogen®) or 1X PCR Master Mix (Promega®). Electrophoregrams were checked by eyes and validated sequences were aligned using Seaview [16].

F. Bioinformatics analysis

Sequences were identified by SAP (Statistical Assignment Package) software [17, 18], a method using Bayesian approach to statistical assignment. The method has advantages compared to the online BLAST search tool, by including phylogenetic information and providing statistically meaningful measures of confidence (posterior probabilities) to the taxonomic assignment.

III. RESULTS AND DISCUSSION

We focused in this study to optimize an ideal DNA extraction method for leathers. Thus, 200 mg of modern chamois leather sample were extracted using the most used DNA extraction methods for degraded DNA, Phenol-Chloroform and silica. The final extract volume for both was 100 μ l. Specific chamois PCR mix were containing increasing extract volume (0,25-2 μ l). With Phenol-chloroform extraction method, the extract was colored, therefore we were obliged to perform extract purification using Minelute Purification Kit (Qiagen®). Furthermore we added Chicken Serum Albumin in the PCR mix. Finally we had amplifications only for 0,25 and 0,5 μ l of extract (Figure. 1).

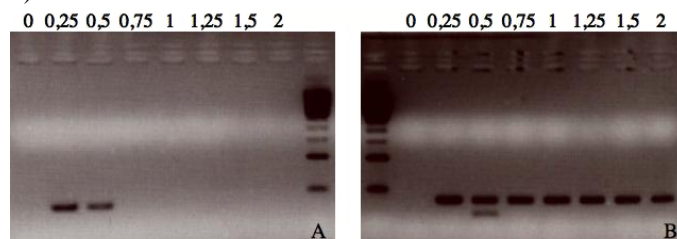


Fig. 1

Reverse image of Ethidium bromide-stained gel of specific PCR products (using specific Chamois primers) of DNA isolated from chamois leather. 0 to 2: extracts volume in μ l added in a PCR tube. A: With Phenol-chloroform extraction method, the extract was colored, therefore we were obliged to perform extract purification using Minelute Purification Kit (Qiagen®). Furthermore we added Chicken Serum Albumin in the PCR mix. B: With Silica-modified method, the extract was colorless. Thus, without any further purification or addition of animal serum in PCR tubes, we obtained amplifications for all volumes of extract.

With Silica-modified method, the extract was colorless. Thus, without any further purification or addition of animal serum in PCR tubes, we obtained amplifications for all volumes of extract. These results have suggested the Silica-modified as non-inhibitive DNA extraction method for species identification in Leather. We realized that the critical

condition of silica extraction method is that the pH must be monitored during all steps. Otherwise DNA is released during washing steps or remains linked to silica particles after elution.

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