Exploring DNA Diversity in Leathers: An Approach on Identification of Origin

by

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Abstract

DNA based approaches have become widespread in recent times to identify the origin of samples when its phenotypic characteristics are not distinguishable. This, in particular, applies to the leather industry wherein with an increase in duplicated embossing of grain patterns; there is a need to detect the animal origin of commercial leather articles. Thus, the characterization of molecular markers that enables rapid detection of the leather source helps us in precise species identification. The present study aims to generate definite sequences between the four major species in the Bovidae family (Buffalo, Cow, Goat, and Sheep), which are the major players in the manufacture of leather products, especially in India. Based on specific mitochondrial sequences, a specific fragment of the mitochondrial 12SrRNA gene was amplified by PCR as a marker for species-level identification. By the maximum homogeneity, from the NCBI and BOLD database, the BLAST analysis of the sequences of amplicons from unknown sources, distinguish closely related species of the subfamilies Bovinae (buffalo and Cow) and Caprinae (sheep and goat) and this 12SrRNA based PCR-BLAST analysis is a good tool to identify the origin and control the quality of leathers that are being manufactured. The present study has optimized an approach for the extraction and amplification of DNA from the finished leather, which is one of the most significant challenges because of the vigorous processes encountered during their manufacture. The findings of the study have commercial value at large scale.

Introduction

Leather, a highly commercial value product of animal skins or hides of the bovidae family subjected to various unit operations as shown in Figure 1. The process involves huge pH variations in order to facilitate the skin protein layers to be stabilized or tanned. Until tanning, the animal origin of the tanned material has been approximately identified based on the size and thickness of the skin or hide. Since the grain pattern of the skin or hide has significant variations, it has also been taken as a tool for the identification of the animal origin.[1] However, when the tanned material is transformed into a finished leather product, the loss in the surface as well as thickness pose challenges in the identification of animal origin. Further, to satisfy the need of the customer on design and pattern, and with the advancement of technologies, the original grain pattern of the skin/ hide has been masked with the required patterns through embossing, which completely affect the identification processes at significant level. Though other chemical and protein analyses have been suggested[2, 4] but it holds good with the untanned material and not for the finished leather, wherein, the protein has been highly stabilized with the tanning agent.

In order to have a reliable and highly precision method to distinguish the animal species of the finished leather products, DNA based paleogenetic approach may provide the solution.[2, 5] The sustainable identification capability is possible with the combination of DNA sequence which provides an efficient and substantial path for species authentication.[6-8] With reference to the finished leather product, since, the leather making process itself eventually affects the survival of DNA (a wide pH variations in the process as detailed in the Figure 1), the DNA profiling of finished leather product[9, 10] is challenging. Similarly, Ojeda et. al.,[11] reported that it is difficult to discriminate the leather manufactured from Caviidae, Tayossuidae and Suidae families after the tanning process. Hence, the authors suggested the molecular approach, which solved the problems associated in identifying the animal origin of the products obtained from the said three families. In addition, the previous literatures have documented the survival of mitochondrial DNA (mt DNA)[12,24] in the 19th century leather articles, and thus provides a meager chance for the molecular traceability of the species in leather product samples and articles[13] and accordingly extracted the mtDNA from the finished leather samples of three families. A report on sheep and goat[2] employed only Chamois leather and a recent report[3] emphasizes the species identification using mtDNA with the amplicon size ranging from 52-75 bp. The results of the said studies may give a solution for the identification issues of leather samples of the bovidae family, however, still more authentication reports are in demand. Since, the finished leather products of the bovidae family have numerous chemicals which affect the extraction of the traceable quantity of mtDNA which in turn might also significantly affect the amplification process.[9]

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Leather from the bovidae family occupies the major place in the Indian leather sector, identification of the species from which the leather has been made is vital in the current scenario to control the fraudulence happening. Hence, comparisons of nucleotide sequences of complete mtDNA or of various regions (for e.g., genes encoding cytochrome b,[14] cytochrome oxidase, 12S rRNAs[15] and 16S rRNAs),[16] find helpful in understanding the phylogenetic relationships between species.[17-21]

Thus, the present study aims to develop a simple and reliable technique to discriminate the bovidae family animal origin of the finished leather product by following the refined DNA extraction procedures, construction of highly specific and sensitive primers, PCR amplification, sequencing of amplified product and authentication of the study with unknown samples. This method may prove to be an efficient method to control the quality of the leather produced, reducing the occurrences of duplications.

Experimental

Sample collection

The finished leather samples for all the four species (Buffalo, Cow, Goat and Sheep), were collected from the Leather process Division of CSIR-Central Leather Research Institute, Adyar, Chennai, Tamil Nadu, India. All the finished leather sample products were tanned with basic chromium sulphate. The thickness/grain-pattern of all the test samples were measured/observed for morphological validation of the species before further investigation.

DNA extraction from leather samples

The collected finished leather product samples were finely chopped to small pieces using sterile scissors and blades. DNA was extracted from leather samples according to the procedure described[11, 22, 23] with few modifications to have better DNA yield. The optimisations to the final DNA extraction method was done after several trial and error runs. The known finished leather samples of 25 mg was placed in a 1.5-mL tube and 500 µL lysis buffer (10-150 mM Tris-HCl, pH 8.0 – 9.0, 10 - 150 mM EDTA, 2.0-3.0% SDS, and 10- 150 mM NaCl) and 20 µL proteinase K (20 mg/mL, GeNei) and 5 µL of Rnase A (10 mg/ml, Thermo scientific™) was added and the samples were incubated at 50°- 60°C in a dry bath for 8 - 12 h with pulse stirring. The general protocols such as centrifugation and the supernatant collection done accordingly to separate the DNA. One µl of the obtained DNA was used for determining DNA concentration and quality in Biotek micro plate reader. The samples were then subjected to electrophoresis on a 0.7% agarose gel in 1X TBE and the presence of DNA was quantified with a gel dock (Medicare Gelstan).

Primer Design

Table II describes the primers specific to the region corresponding to mitochondrial 12S rRNA obtained from *in silico*. All the published sequences of the four animals were collected from NCBI genbank and performed the multiple sequence alignment using Clustal W (EMBL/EBI). Primers were designed for each Genus according to the conserved region within the genus and hypervariable between genus. Although there were many priming sites, the primers that correspond to in silico amplification and no cross reactivity of all the four selected sequences were selected. The designed specific target gene primers were synthesized (Eurofins Genomics India Pvt Ltd). Further, to ensure the specificity of the designed primers, we amplified the primers on the freshly extracted DNA. The amplicons were extracted, sequenced and analyzed using NCBI Blastn database. The sequencing results confirmed the specificity of the designed primers by only amplifying the respective target gene of the corresponding animals and does not have a cross reactivity.

PCR Amplification of Control and Test samples

Thermal Cycling was conducted with 1U of Taq DNA polymerase in a 25-µl reaction mixture, consisting of 50 ng of genomic DNA, 1.5 µM of each primer (Eurofins Genomics India Pvt Ltd), 200 µM deoxyribonucleotide triphosphates (dNTPs) and 1X Taq buffer (Bangalore Genei, India) with 1.5mM Mgcl2. With the prepared template and primers, PCR was conducted using the following temperature profile: initial denaturation at 95°C for 3 min, then 35 cycles of 45 Sec at 94°C, 45 Sec at 54.3°C, and 45 Sec at 72°C; and finally, an extension reaction of 3 min at 72°C. The eppendorf Thermal cycler (Eppendorf North America Inc) was used for PCR reaction. In the present study, the normal Taq polymerase was used whereas the hot start Taq polymerase may be used in future experiments for the betterments in the amplification specificity.

For test runs, both the quantity of DNA and the cycle runs were varied to have the optimum conditions. DNA quantity ranging from 1 to 100 ng and the cycle numbers were of 30, 35, 40, 45. Every sample was amplified with respective primers twice to check the reproducibility. The amplified products were electrophoresed with DNA ladder (Step UpTM 100bp DNA Ladder, Bangalore Genei, India) on 2 % agarose gel. The amplified PCR bands were subsequently visualized in UV transilluminator for the amplified fragment size validation (Gelstan 1612, India).

DNA sequencing

The PCR products were eluted and purified using QIAquick Gel Extraction Kit (Qiagen, Inc. Valencia, CA, USA). Sequencing was performed on 3500 Genetic Analyzer, Applied Biosystems (CA, USA) using the Big DyeTM terminator cycle sequencing ready reaction mixture in accordance with manufacturer's instructions. The sequences retrieved were analyzed by sequencing analysis software 6 v6.0, Applied Biosystems (CA, USA).

The expected amplified DNA band for all the test samples were excised from gel and purified using the QIAquick Gel Extraction

Kit (Qiagen, Inc. Valencia, CA, USA) as per the manufacturer's protocol. Sequencing reactions were carried out with a BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) using the PCR-primers as sequencing primers by using ABI Prism 3500 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The sequences thus obtained were assembled and edited using Sequencing analysis software 6 v6.0, Applied Biosystems (CA, USA).

Authentication of the method

In order to further authenticate the protocol and the primers designed for the Bovidae family, a double-blind study was conducted using unknown (test) finished leather samples received from the tanning industries. Further, a skin sample received from aquatic species has been considered as a negative reference.

Bioinformatics Analysis

Identification using BLAST

Obtained sequences were run for BLAST search against public database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?LINK_LOC=blasthome&PAGE_TYPE=BlastSearch&PROGRAM=blastn) to determine the closest match of unknown specimens.

Results and Discussion

Morphological analysis

Figure 1a illustrates the steps involved in the leather manufacturing process from animal skin/hide of the bovidae family along with the morphology of the samples obtained from each step for a representative animal skin. Figure 2a illustrates the representative

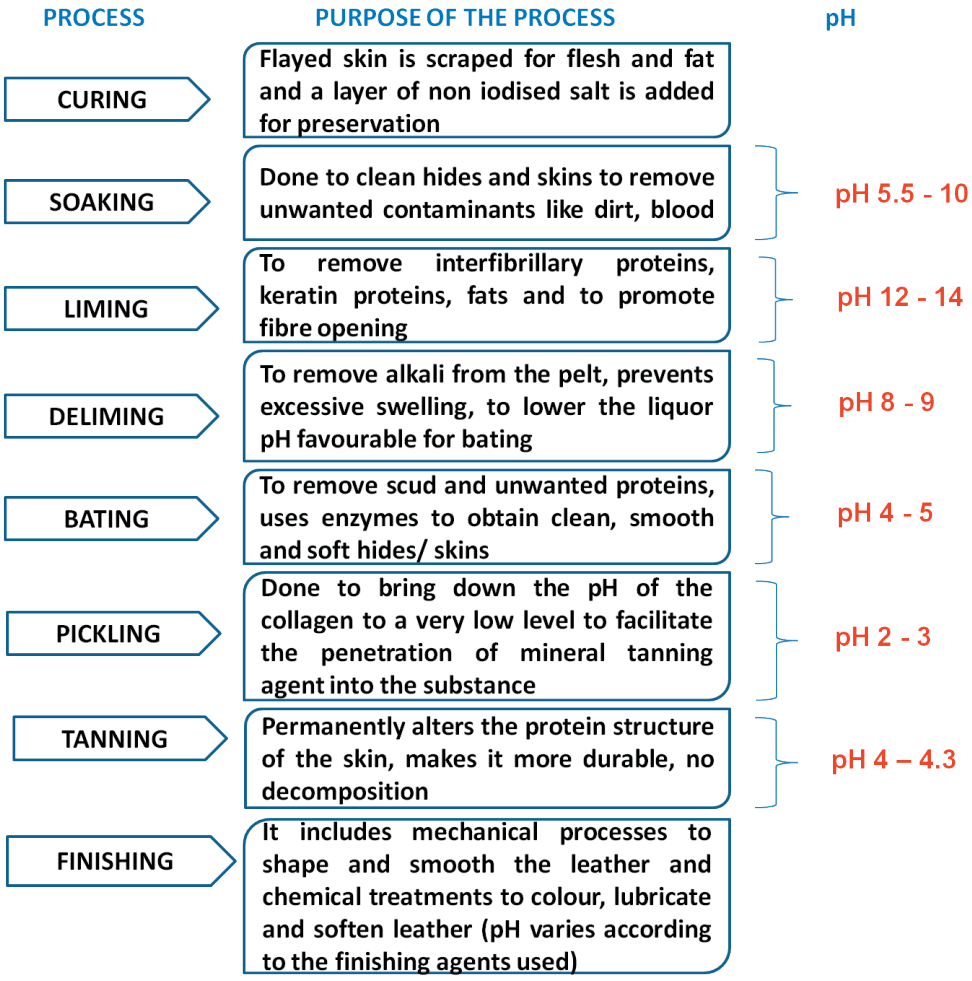


Figure 1. Schematic representation of unit operations of leather processing wherein the animal skin/hide has been transformed to finished leather. The pH variations in each unit operations were described in the figure. In brief, during the leather production, the skins and hides are subjected to various pH conditions which plays a significant role in the stability of the nucleic acids. These pH conditions lead to the degradation of the DNA present in the skin/hide thereby the survival rate of nucleic acids are very negligible. Therefore, to increase the rate of success in PCR, mtDNA was used as a template in the amplification in the present study. As the copy number of the mtDNA was higher than that of the autosomal DNA (Each cell contains 2 copies of nuclear DNA while the same cell contains 100 - 10000 mitochondrial genomes) mitochondrial DNA is preferred as template in PCR.

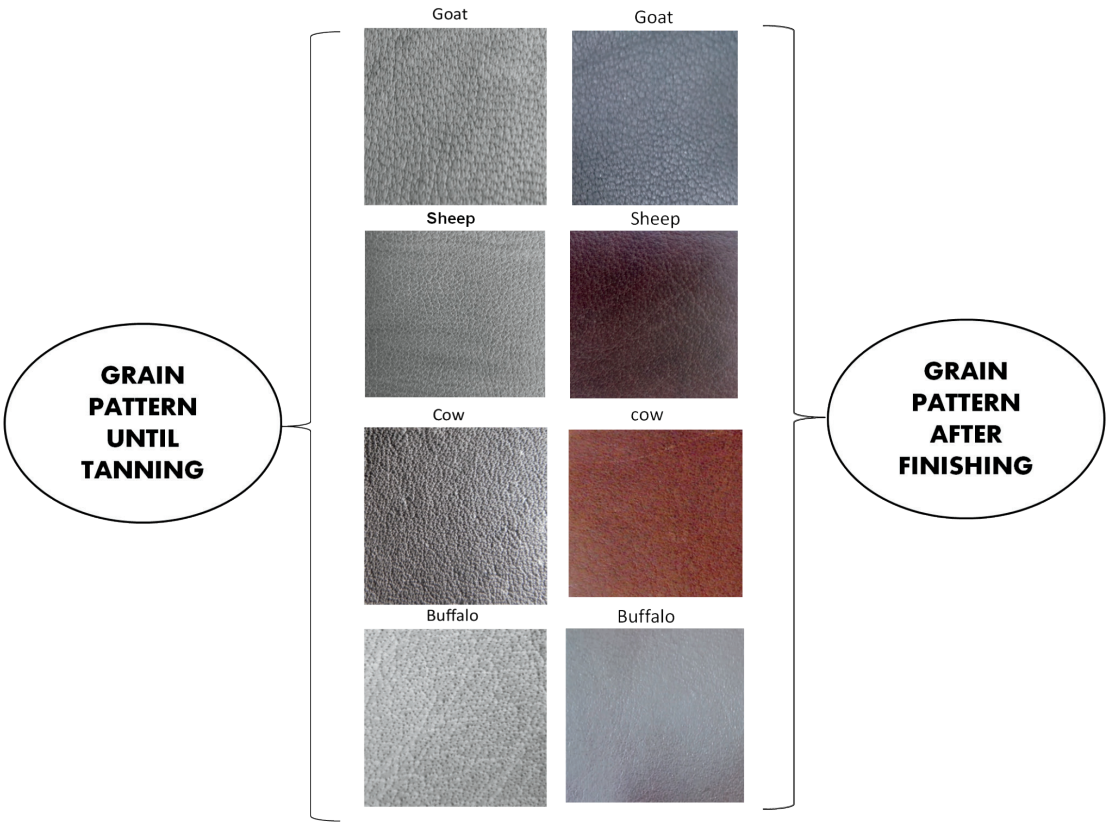
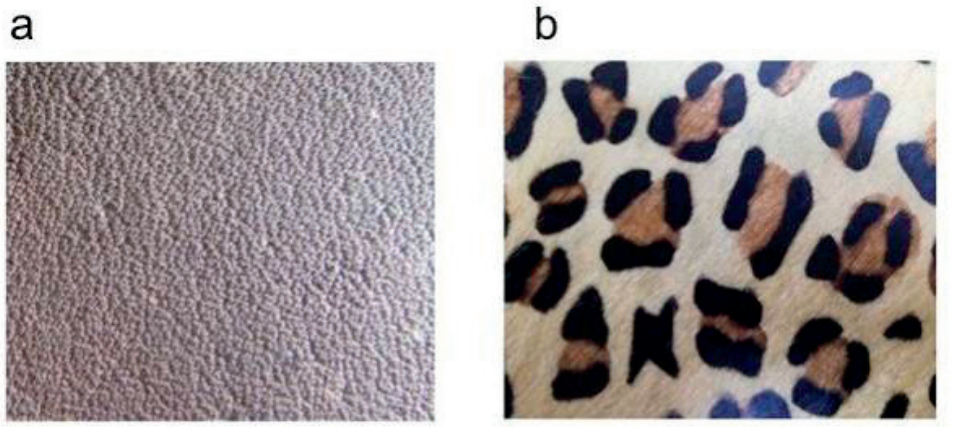


Figure 2a. Representative image on the comparison of Grain patterns of the leather samples of bovidae family until tanning and after finishing. The image indicates the changes in the morphological characteristics of the grain pattern present in the leather during pre-tanning and post -tanning operations in leather making process.



(a) - Cow finished leather (hair free tanning process) grain pattern – optical view
(b) - Cow finished leather (hair on tanning process) with leopard pattern

Figure 2b. A representative image on the embossing of leopard pattern on the hair on tanning finished cow leather. The image depicts an illustration on how a common end user cannot distinguish the cow leather and a leopard leather. Visual inspection may not correctly identify the species of leather at every time, hence, further authentication involving molecular techniques is a necessity.

Table I

Comparison on thickness profile of the animal skin of the Bovidae family until tanning and after finishing

S. NO	Species	Thickness of the material (mm)	
		Until tanning	After finishing*
1	Cow (Calf/Hide)	1-6	0.9 -3
2	Buffalo (Calf/hide)	1-8	1-3.5
3	Sheep skin	0.5-2	0.5-1.5
4	Goat skin	0.5-2	0.5-1.5

(*Finishing involves, shaving, splitting, dyeing, fatliquouring, setting, staking, buffing and toggling. Splitting of leathers for the requisite purposes may have different thicknesses).

image on the grain pattern of skin/hide samples before and after finishing. Figure 2b illustrates the embossing of leopard pattern on the cow finished leather, wherein the process of cow skin/hide involves hair on tanning. Table I depicts the thickness profile of the animal skins of the bovidae family untill tanning and after finishing.

As described, the finished leather samples have different thickness and have embossed patterns and have been considered as the major challenges in the identification of animal origin. The rigorous manufacturing steps as described in Figure 1 suggests the wide pH variations in each step. During the first step, the skin/hide samples have been exposed to high alkaline pH which support the removal of the hair and flesh in the presence of reducing agents and then exposed to acidic pH which supports the tanning process and again brought down the pH to near neutral during the post tanning operations which includes, dyeing, fatliquoring, etc. for the better quality and organoleptic properties. The finished leather samples thus obtained may or may not have macromolecules like DNA and if at all may be in very meager quantity. Hence, the prime task of the present study is to recover the low abundance of mtDNA and also to maintain the quality of DNA from the finished leather samples of the bovidae family by cost effective methods. The extraction process does not involve phenol –chloroform or guanidinium thiocyanate/ silica. But, the process employs modified protocol of Ojeda et al.[11]

DNA extraction from finished leather samples

The DNA extracted from the known animal origin of finished leather products of the bovidae family suggested the reliability and the precision of the procedure followed in the present study. The method yielded plenty of good quality of DNA required for the present study.

PCR amplification and DNA sequencing of control and test samples

Table II describes the species specific primers to the region corresponding to mitochondrial 12S rRNA obtained from *in silico*. All the animals included in the study belong to the Bovidae family, so designing a species specific primer set with short amplicon size was not possible as they are closely related. Figure 4 (a-d) depicts the PCR amplification profile of four finished leather samples chosen for the present study. The amplicon size for respective animals were identified as 331 bp (Goat), 356 bp (Sheep), 280 bp (Cow) and 451 bp (Buffalo) which matches with the primers as designed inaccordingly. Figure 5 (a-d) illustrates the sequencing chromatogram of all the four known animal origin DNA samples. It has been observed that all the four samples showed more than 75% of good nucleotide reads (shown in blue).

Following the assessment on known leather samples, experiments were conducted with the unknown samples (10 numbers) received from tanning industries in Tamil Nadu, India, which includes leather from aquatic animals (Fish). These test samples upon extraction of DNA as per the modified procedure described in the present study were then amplified with the designed primers. Figure 6 depicts the PCR amplification profile of the test samples. It has been observed that out of 10 samples (S1-S10), the amplicons of the four samples matches with the sheep (356bp) and two of the amplicons matches with the cow (280 bp) and one amplicon resembles Buffalo (451 bp) and the remaining two samples matches with Goat (331bp). The last lane (S10) displayed the absence of the polymerized amplicon which suggested and authenticated that the origin of the sample is different from the bovidae family.

Table II

Details on primers of specific region chosen for the animals of bovidae family for identification

Primer ID*	Sequence	Basepair length	Target
Goat	5' - AGCCCGAAACTCAAAGGACT - 3'	331	12S rRNA mtDNA
	5' - CGTGCTTCATGGCCTAATTC - 3'		
Sheep	5' - AGCCCGAAACTCAAAGGACT - 3'	356	12S rRNA mtDNA
	5' - CGTGCTTCATGGCCTAATTC - 3'		
Cow	5' - ACCGCGGTCATACGATTAAC - 3'	280	12S rRNA mtDNA
	5' - CGCCAAGTCCTTTGAGTTTT - 3'		
Buffalo	5' - CAAACTGGGATTAGATACCCCACTAT- 3'	451	12S rRNA mtDNA
	5' - AGGGTGACGGGCGGTGTGT - 3'		

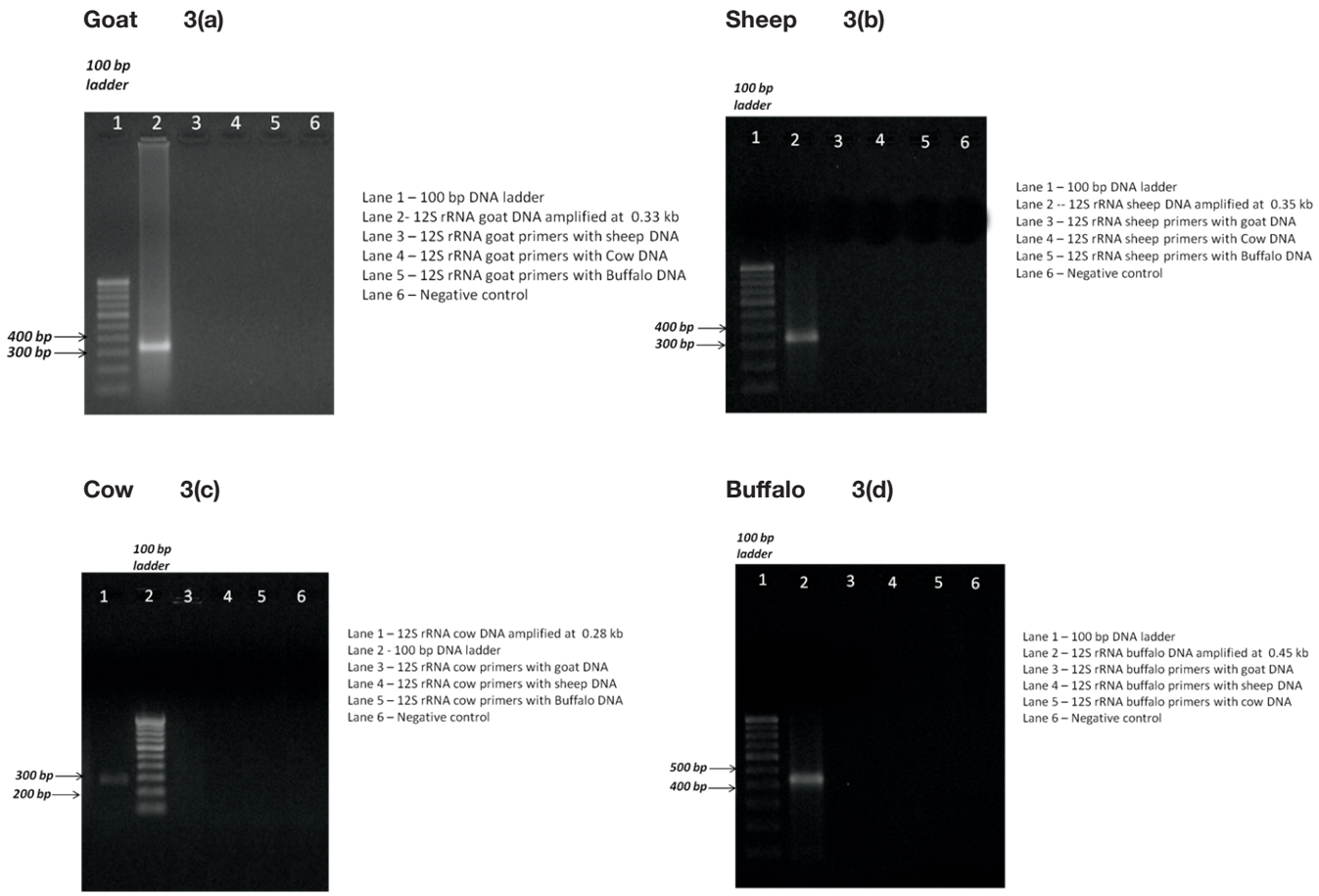


Figure 3 (a-d). PCR amplification profile of the four finished leather samples of Bovidae family, indicating amplification of Goat, Sheep, Cow and buffalo at respective basepairs, viz., 0.33 kb, 0.35 kb, 0.28 kb, 0.45 kb.

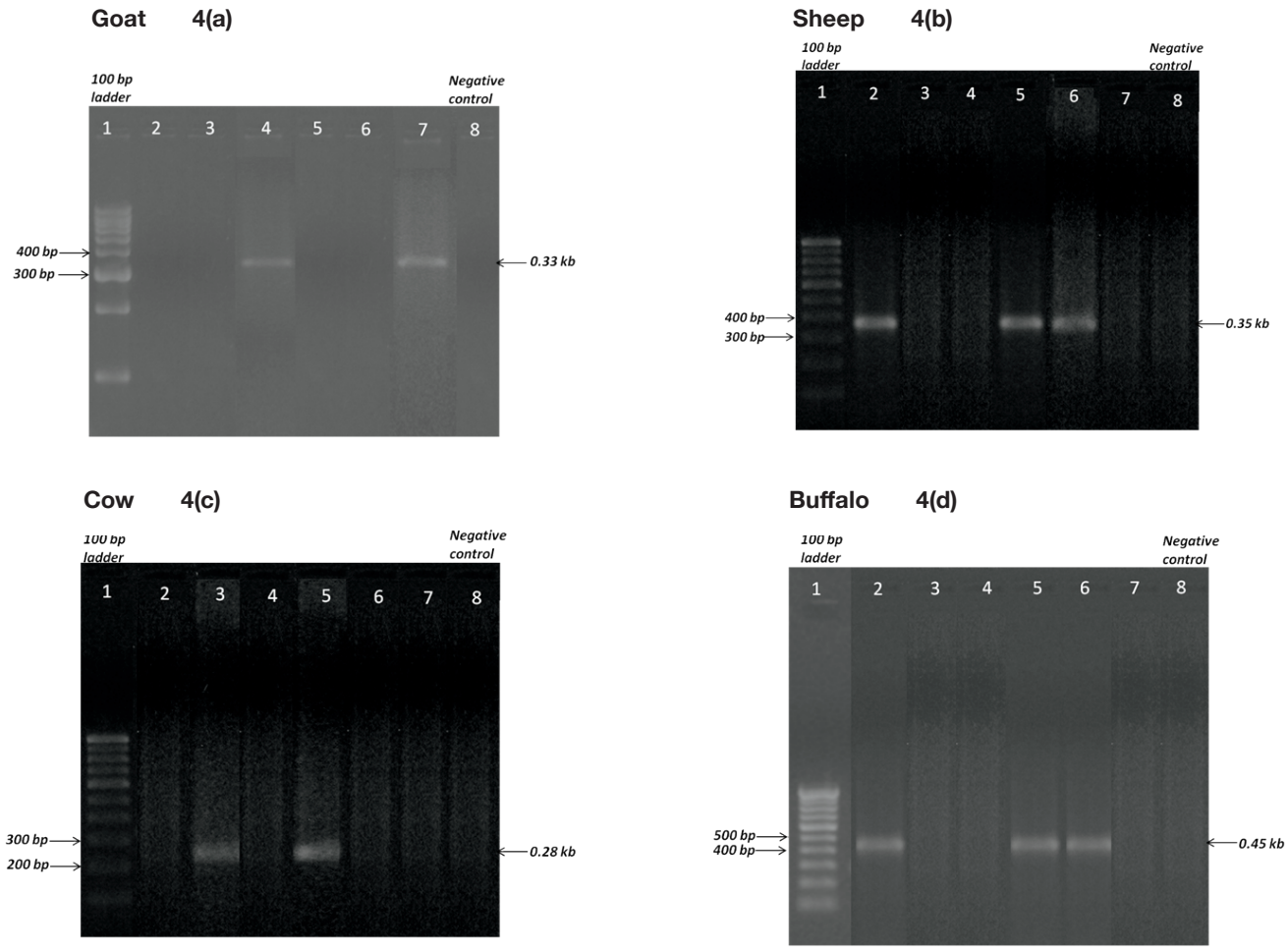


Figure 4 (a-d). PCR amplification profile of the test (unknown) samples. In fig 4a, the amplicons of the two samples matches with the Goat (331bp) while in fig 4b, the amplicons of the three samples matches with sheep (356bp). The fig 4c, represents the two amplicons that matches with the cow (280 bp) and the fig 4d, illustrates three amplicons that resembles Buffalo (451 bp). The lane 8 in all the four animals represent the negative control.

The present study on identifying the animal origin of the finished leather samples of bovidae family of Indian origin, suggested that with less survival of macromolecules (DNA), it is possible to extract the mtDNA from the finished leather samples using the modified method. Results on PCR amplification studies on known (control) and unknown (test) samples along with a negative reference sample (no template) evidently proved that the primers designed based on the conserved sequences are so specific to the species and are able to amplify the targeted gene without any cross-reactivity. DNA based species identification does not require haplotype sharing. Moreover, this problem did not occur with 12S rRNA gene as its evolutionary rate is higher. The success rate of the statistical identification of species is high up to 99% with respect to 12S rRNA. The variable priming sites of 12S rRNA could be a great tool for identifying the source of an unknown leather sample, which comes under the Bovidae family, by BLAST analysis.

Further, the future study relies on the development of multiplex PCR or PCR-RFLP to ease the identification for the test samples. Instead of using Sequencing reaction which is expensive and laborious; multiplex PCR or PCR-RFLP methods will be a reliable alternative and provide a rapid, simple, easy method of identifying the leather origin. Thus, the present study finds an immense value to the leather sector.

Sequence Submission to Genbank Database

The sequences retrieved were directly submitted to NCBI Genbank Database and received the Accession numbers as KX824125, KX824126, KX824127, KX824128.

Conclusions

In the present study, we have shown that the unknown (test) leather samples can be detected for its origin using the designed species specific 12S rRNA primers of the bovidae family. Also challenges faced during the isolation of DNA from leather samples were also discussed. Indeed, results of the present study have proved that the 12S rRNA based PCR- BLAST is one of the simplest and easiest methods for the detection of the leather sample origin and also helps in finding the fraudulence among the leather samples.

Conflicts of interest

There are no conflicts to declare

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