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Domestic animals' identification using PCR-RFLP analysis of cytochrome b gene

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Abstract

Background: Species identification is an important process to identify the origin of meat, adulteration and for cooked and processed meat. The present study was conducted to identify cattle (*Bos taurus*) and buffalo (*Bubalus bubalis*) by using mitochondrial cytochrome-b (*Cyt-b*) gene. Size of the gene is 1140 bp, but we amplified 359 bp that is cleaved by specific restriction endonucleases. The aim of this study was species identification through *Cyt-b* gene by using PCR-RFLP analysis.

Methods: For this study, 55 blood samples were collected from different species of domestic animals. The DNA was extracted from the whole blood through blood extraction kit. The DNA of these samples were amplified through PCR using universal *Cyt-b* primers. The amplified product was treated with restriction enzymes *Alu I*. The resultant fragments were viewed on 3.0 % agarose gel.

Results: *Cyt-b* gene was amplified of all included animals. Different bands were observed as compared with 50 bp DNA ladder. Animals were identified on the base RFLP mediated by *Alu1* restriction enzyme.

Conclusion: We identified domestic animals on the basis of Mitochondrial *Cyt-b* gene by the process of PCR-RFLP. To identify specific animals through RFLP, a larger sample size and confirmation by gene sequence analysis may be helpful.

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Introduction

Meat and meat product is considered an important source of food throughout the world. Adulteration of the meat is very serious issue to be considered for consumers [1,2]. There is also fear of disease spreading and risk to public health. Animal species identification is useful in solving criminal problems, kinship cases, maternity and paternity testing, lineage, wildlife trafficking, illegal trade, inbreeding counting, speciation, and for protection of various animal lives [3,4]. Currently for meat testing different techniques are used in which the most common is PCR-RFLP. In forensic field PCR–RFLP is very important technique and commonly used analysis for genetic identification of various species [5,6]. Most of the species identification is done through mitochondrial genes as compared to nuclear genes based on sequences diversity because mitochondrial genome having less chance of mutation as compare to nuclear genome [7]. There are more than one mitochondrial genes used for different species identification like 16S and 12S rRNA but the most common is cytochrome-b (*Cyt-b)* gene which has been in use for the last 2 decades for the animals identification to differentiate among nearly related taxa [8-10]. *Cyt-b* gene length is 1140 bp; it has different stable and proper sequences which are used for the suggestion of various universal primers, and also variable sequences which are useful for identification of animals. But usually the whole gene is not amplified because it is time consuming and difficult process, we amplify short fragment of the gene like 402 bp or 359 bp in some cases which is cleaved by specific restriction endonucleases like *Alu I* [11,12]. *Cyt-b* gene shows high polymorphisms which play a key role in species identification. Species identification studies based on *Cyt-b* have ranged from both short (400bp) to long (900bp) PCR-RFLP, DNA sequencing, and variable size-species specific multiplex PCR [4-6,9,10]. The aim of the study was the identification of domestic animals' species through *Cyt-b* by using PCR-RFLP.

Methods

Sample collection

Total of 55 blood samples were collected from different areas of the District Swat including 3 blood samples from each species of broiler chicken, cat, donkey, goat, horse, sheep, rabbit; 2 from turkey; 4 from dogs; 9 from buffalos and 19 from cattle. The blood samples from domestic organisms were collected in EDTA tubes using 3cc or 5cc syringes by the permission of the owner of domestic animals. The blood samples were stored in the refrigerator in Forensic Research Laboratory at University of Swat before further processing.

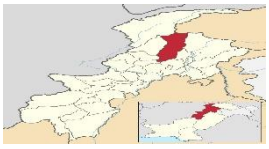

Figure 1: Map of District Swat, Khyber Pakhtunkhwa Pakistan

DNA extraction

Whole DNA contains nuclear and mitochondrial DNA which was extracted from whole blood 55 samples by using Wiz prep genomic DNA mini kit (WIZBIO Solutions, South Korea). Concentration and integrity of the extracted DNA was checked using 0.8% agarose gel.

PCR amplification

To make the concentration of DNA uniform, 150µl 1X TE buffer to the 12 samples (Bright) and added 50 µl to 4 samples and stored at room temperature. The following primers (table 1) were used to amplify a 359 bp fragment of the *Cyt-b* gene.

Cytochrome b	Primer sequence (5´- 3´)	Length (bp)	Length of PCR product (bp)	Ref.
Forward Primer	CCATCCAACATCTCAGCATGATGAAA	26	359	[7]
Reverse Primer	GCCCCTCAGAATGATATTTGTCCTCA	26		

Table 1: *Cyt-b* primer for domestic animals' identification

The amplification reaction was carried out in by PCR Machine (*PERKIN ELMER* GeneAmp PCR system 2400, USA). Total of 25uL Reaction mix was prepared using 5µL of template DNA, 2µl forward and reverse primer each, 15 µl Green Master mix and 6µl deionized water. PCR program was set for DNA samples as: Initial denaturation at 94ºC for 5 min, followed by 33 cycles of denaturation at 94ºC for 40 Sec, annealing at 56ºC for 40 sec and extension at 72ºC for 40 sec and subsequent final extension at 72ºC for 5 min.

RFLP analysis

RFLP analysis was carried out by adding specific restriction endonucleases *(AluI)* to PCR products which resulted different bands for each individual on 3.0% agarose gel.

Requirements	Volume (µl)
PCR reaction mixture	10
Nuclease free water	18
10X Buffer Tango	2.0
AluI	1-2

Table 2: Reagents composition for RFLP analysis

The above ingredients were mixed gently and spun down for a few seconds then incubated at 37°C for 1-16 hours. The RFLP samples were electrophoresed using 3% agarose gel. Fifteen microliters of each sample were loaded in the gel and run for 90 minutes at 100V.

Results

The extracted DNA samples were run on 0.8% agarose gel that confirmed DNA bands (figure 2).

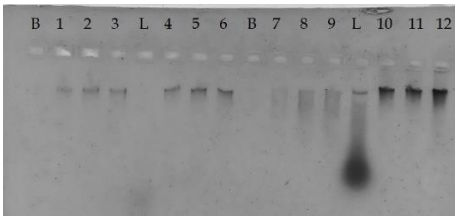

Figure 2: Gel electrophoresis of extracted DNA samples.

PCR amplicons were run on gel that showed different bands when visualized using gel documentation system.

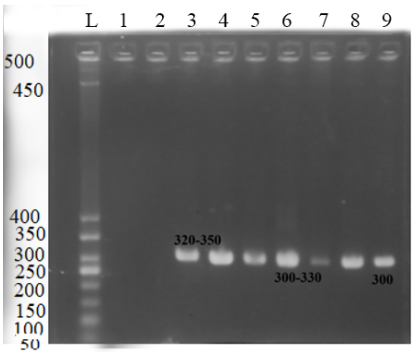

Figure 3: shows different bands for *Cyt-b* to identify many domestic animals. L: Ladder, Well 1 and 2: Blank, Well 3 and 4: Buffalo, Well 5: Bull, Well 6: Dog, Well 7: Sheep, Well 8: Turkey and Well 9: Chicken.

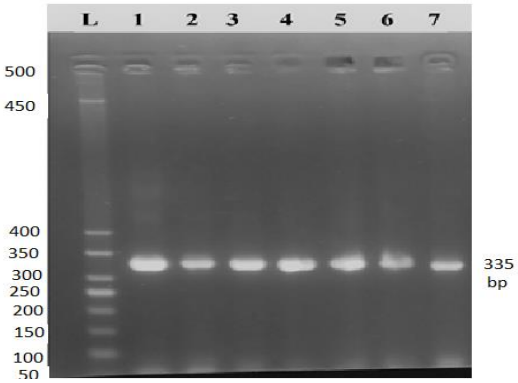

Figure 4: Amplification product of *Cyt-b* gene in 7 studied sample L: 50 bp DNA ladder, Well 1: Bull, Well 2, 3 and 4: Buffalo, Well 5: Rabbit and Well 6 and 7: Horse.

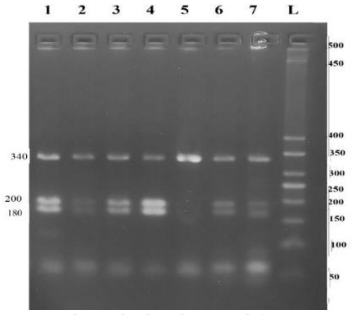

Figure 5: PCR-RFLP based *AluI* digested fragments of *Cyt-b* gene in study samples: L: ladder, Well 1: Bull, Well 2, 3 and 4: Buffalo, Well 5: Rabbit, Well 6 and 7: Horse. RFLP analysis for domestic animals that showed 3 fragments in bull, buffalo and horse; and 1 fragment in rabbit.

Discussion

In the present study we used *Cyt-b* gene cleaved by a single restriction endonuclease *AluI*, which generate different fragments for each individual like 340, 200, and 180 bp in bull and buffalo; 340 bp in rabbit; and 340, 200, 180 bp in horse. Species identification is carried out by using *Cyt-b* gene through PCR-RFLP. Different enzymes have been reported in the literature like *Bst*UI, *Taq*I, *Rsa*I, *BsaJ*I, *Alu*I, *Nsi*I, and *Bst*NI and *Hinf*I [13]. Using PCR-RFLP technique, variation in *Cyt-b* fragments have been identified in different species such as buffalo, cattle, rabbit and horse. Previously a study was aimed to develop a simple method for identification of various species through specific primers for amplification of *Cyt-b* gene in mitochondrial DNA. Another study was conducted where DNA from blood sample was amplified through *Cyt-b* gene primers followed by the process of RFLP which showed two fragments in buffalo (290 and 68 bp) and two in sheep (312 and 46 bp) for *Alu I* restriction enzymes. For the amplification of *Cyt-b* gene universal primers are used (H15173 and of L14816) to identify sheep buffalo and camels [14]. A single fragment with a size of 359 bp resulted from PCR amplification of the *Cyt-b* gene in cattle and buffalo. Our indings are in sync with this study because the volume of PCR is almost similar to each other. Fragments number and size difference is because of differences in the primers used.

Elsewhere in the literature, RFLP analysis of the *Cyt-b* gene fragment for three different animal species revealed high specificity and discrimination, with no common fragment shared among the studied species. Moreover the resulting fragments were the same for each species in all samples regardless the age (newborn, young and adult) [14]. 359 bp long fragment was digested by restriction enzyme *AluI* to specie specific patterns. Results of digestion were visualized after electrophoresis on 3% agarose gel. PCR product of rabbit (*Oryctolagus cuninculus*) did not have restriction place for *AluI*, so the whole 359 bp long fragment is visible on agarose gel.

Main focus of our research was to identify domestic animals through *AluI* restriction enzyme which showed bands differentiation in animals. We compared different research with our work that shows similarities because we used same gene for identification purposes. All these results in the literature as well as our study shows that multiple genes and different primers are used for species identification but most accurate results are shown *Cyt-b* gene using universal primers. Species identification through *Cyt-b* gene for cattle mostly shows 3 fragments and rabbit shows usually single fragment for identification if treated with *AluI* enzyme.

We identified domestic animals on the basis of Mitochondrial *Cyt-b* gene by the process of PCR-RFLP. To identifiy specific animals through RFLP this samples size is less. In future, a larger sample size with more enzymes is needed to predict accurate identification of animals followed by gene sequence analysis.

Conflict of Interest Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contribution

ZU, SGA and MN carried out field and bench work. MAR and MH helped in analysis of data and writing of manuscript. MI and AR coneived the study, carried analysis and helped in write up.

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