Mitochondrial 16S rRNA gene-dependent Blood typing as a Forensic Tool

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Abstract

Background: Mitochondrial DNA is an important tool for human identification and is used to differentiate between human and animal blood at the crime scene, because in extreme conditions nuclear DNA is severely destroyed while Mitochondrial DNA contains multiple copies (200-2000) in per cell as well as resists harsh and more stable conditions. **Methodology:** Seventy-two blood samples were collected from human (*Homo sapiens*), sheep (Ovis aries), goat (*Capra hircus*) and cow (*Bos taurus*) (Eighteen blood samples for each). All blood samples were withdrawn by technician and 5ml were aspirated using aseptic technique and transferred to EDTA-Na2 tube and mixed well and stored in refrigerator. The collection takes 2 weeks (15th May 2019 to 30th May 2019). All samples were collected from Al-Diwanyia city. **Results:** The results of PCR reveal that, the primer pairs were specific and non-specific products not appear for all samples. The amplification of *Homo sapiens* mitochondrial DNA with primer pairs of other (*Ovis aries, Capra hircus and Bos taurus*) and amplification of each with primers pair of another genus gave negative results and this a primary evidence for primer pairs specificity. The amplicon of 16S rRNA gene of Homo sapiens were 1200bp ,*Ovis aries* were 1060bp ,Capra hircus were 820bp , and *Bos taurus* were 1300bp. The sequencing revealed that no cross-reactivity of designed primer pairs and the PCR assay based on the designed primer pairs will be simple, fast, sensitive, specific, and cost-effective. **Conclusion:** Sensitivity, specificity and accuracy of the designed species specific primer pairs and applicability of the designed primer pairs in forensics to investigate blood sports or evidence belonging for human, sheep, goat and cow.

Keywords: *Homo sapiens*, Ovis aries, *Capra hircus*, *Bos taurus*, Forensic.

Introduction

Mitochondria have their possess small spherical genome, mtDNA, which encodes for the thirteen important subunits of the electron transport chain and ATP synthase together with 22 tRNAs and 2 rRNAs necessary for mitochondrial protein synthesis[1,2]. Mitochondrial DNA presents several characteristics valuable used for forensic studies, especially attendant to the absence of recombination, to a great copy number, and to matrilineal inheritance. Mitochondrial DNA typing founded on sequences of the control region otherwise filled genomic sequence is used to examine a variation of forensic mtDNA profiling methods used for human proof of identity and present their use in the chief cases of human identification from non-human [3-5]. Mitochondrial markers that are used for species identification are as follows: *cytb* gene, cytochrome c oxidase subunit I gene, 12S and 16S rRNA segment and control region in wildlife[6-8]. A short fragment of the 12S rDNA was employed for DNA amplification leading to species identification. The mitochondria DNA 16S rRNA gene is an advanced genetic marker for animal genetic diversity. Utilizing gene mitochondrial DNA 16S rRNA. Polymorphism sites, nucleotide variation, and haplotype variety were determined using whole sequences of the mitochondria DNA 16S rDNA gene[9,10]. Animal mitochondrial DNA (mtDNA) is commonly described as a small, circular molecule that

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is conserved in size, gene content, and organization [11]. The aim of this study is to design vaulable species specific-PCR tool to distriminate blood of human and non human Species specific- primer design.

Methodology

Study Design

The study design was experimental to design species specific primer pairs for typing the blood samples and their assignment to human (*Homo sapiens*), sheep (*Ovis aries*), goat (Capra hircus) and cow (**Bos taurus**).

Blood Sample Collection:

Seventy two blood samples were collected from human, sheep, goat and cow (Eighteen blood samples for each). All blood samples were withdrawn by technician and 5ml were aspirated using aseptic technique and transferred to EDTA-Na2 tube and mixed well and stored in refrigerator. The collection take 2 weeks (15th May 2019 to 30th May 2019). All samples were collected from Al-Diwanya city.

Primer Design

The gene selected for this study is mitochondrial 16S rRNA gene. the NCBI data base were used to recover the sequences chosen for primer design. The sequence ID of *Homo sapiens*:(NC_012920.1); sequence ID of *Ovis aries*: (NC_001941.1); sequence ID of *Capra hircus* (NC_005044.2); sequence ID of *Bos taurus* (NC_006853.1). Primer 3 software [12] was used to design the specific primer using the sequence of above mentioned sequences ID. The generated primer were: Homo 16S-F: GCCTGGTGATAGCTGGTTGT, Homo 16S-R: ATCATTTACGGGGGAAGGCG (1200bp); Ovis 16S-F: AGGCCTAAAAGCAGCCATCA, Ovis 16S-R: GCCCTTTTCTAGGGCAGGTT (1060bp); Capra 16S-F:GCCTGGTGATAGCTGGTTGT, Capra 16S-R: TCACCCCAACCAAAACTGCT (820bp) and Bos 16S-F: CTAAGCAGCCCGAAACCAGA, Bos 16S-R: GGGCAGGGTTTTGTGTTGTC (1300bp).

Mitochondrial DNA extraction

G-spinTM Total DNA Extraction Kit(50 Preps) (REF: 17045) was used to extract mitochondrial DNA from blood of different species according to the manufacturer's protocol instructions.

Agarose gel electrophoresis

Agarose gel was prepared by dissolving agarose powder in 1X TBE buffer. The amount of agarose which can be dissolved depending upon the purpose in which agarose sheet used. 0.7% agarose gel used for visualization the DNA after extraction while 1.5%-2% agarose sheet visualization of PCR product (amplicon). RedSafe (alternative for ethidium bromide) stock solution concentration was 10 mg/ml. Only 5µl of RedSafe stock solution were added to 100ml of melted agarose gel to get final concentration 0.5µg/ml [13,14].

Primer pairs preparation and PCR conditions

The primers were synthesized at (Macrogen/ Korea), were provided in a lyophilized from, which were re-dissolved with 300 nuclease-free water according to institution of manufacture company to reach to the final concentration (100 pmoles/µl). The working solution will be 10 pmoles/µl to be used directly in PCR[15,16]. The PCR conditions were calculated using online Protocol Optimize writer software. The conditions were illustrated in table (1).

Table (1): PCR conditions

Primer	Conditions			References
Homo 16S-F Homo 16S-R	1	95°C	2min.	This study
	30	95°C	30sec.	
		59.3°C	30sec.	
		72°C	130sec.	
	1	72°C	5min.	

Cont... **Table (1): PCR conditions**

Ovis 16S-F Ovis 16S-R	1	95°C	2min.	This study
	30	95°C	30sec.	
		58.3°C	30sec.	
		72°C	130sec.	
	1	72°C	5min.	
Capra 16S-F Capra 16S-R	1	95°C	2min.	This study
	30	95°C	30sec.	
		58.3°C	30sec.	
		72°C	130sec.	
	1	72°C	5min.	
Bos 16S-F Bos 16S-R	1	95°C	2min.	This study
	30	95°C	30sec.	
		59.3°C	30sec.	
		72°C	130sec.	
	1	72°C	5min.	

Result and Discussion

The four sets of designed primer pairs were submitted to specificity using Primer-Blast and the results revealed that, they are specific to amplify 16S rRNA gene of Human *(Homo sapiens*), Sheep (*Ovis aries*), Goat (*Capra hircus*) and Cows (*Bos taurus*) (table 2). 16S rDNA region is highly conserved region among mtDNA[17]. mtDNA can be easier to retrieve from low-quantity and/or degraded DNA samples, as it is present at many copies per cell, thus providing a clear advantage over nuclear genome-based methods of species identification[18-20].

Table (2): Primer-Blast of designed primer pairs

Gene	**Primer Sequence 5' to 3'**	**Sequence ID of isolate**	**Identity**
Homo sapiens 16S rRNA	F:GCCTGGTGATAGCTGGTTGT R:ATCATTTACGGGGGAAGGCG	MN115376.1 MN053904.1 MN125706.1 MN163828.1 MN163832.1 MN125705.1 MN163282.1 MN125704.1 MN124446.1 MK069579.1	100%

Cont... **Table (2): Primer-Blast of designed primer pairs**

Ovis aries 16S rRNA	F:AGGCCTAAAAGCAGCCATCA R:GCCCTTTTCTAGGGCAGGTT	KP998473.1 KP998472.1 KP998470.1 KP702285.1 MH841968.1 MH841967.1 MH841966.1 MG837554.1 MG837553.1 KU681224.1	100%
Capra hircus 16S rRNA	F:GCCTGGTGATAGCTGGTTGT R:TCACCCCAACCAAAACTGCT	LS992662.1 LS992661.1 LS992659.1 LS992658.1 LS992656.1 LS992655.1 LS992654.1 LS992653.1 LS992652.1 LS992651.1	100%
Bos taurus 16S rRNA	F:CTAAGCAGCCCGAAACCAGA R:GGGCAGGGTTTTGTGTTGTC	EU177866.1 EU177865.1 EU177864.1 EU177863.1 EU177862.1 EU177861.1 EU177860.1 EU177859.1 EU177858.1 EU177856.1	100%

The results of PCR reveal that, the primer pairs were specific and non-specific products not appear for all samples. The amplification of *Homo sapiens* mtDNA with primer pairs of other (*Ovis aries, Capra hircus and Bos taurus*) and amplification of each with primers pair of another genus gave negative results and this a primary evidence for primer pairs specificity. The amplicon of 16S rRNA gene of Homo sapiens were 1200bp (Figure 1A), *Ovis aries* were 1060bp(Figure 1B), Capra hircus were 820bp (Figure 1C), and *Bos taurus* were 1300bp (Figure 1D). PCR amplification and sequence analysis of mitochondrial 16S rRNA gene for their use in differentiation/identification and subsequently evaluating their application in solving the forensic cases [21]. Mitochondrial 16S is suitable for the differentiation of 300 mammalian species. 16S rDNA gene is common mitochondrial gene for detection of blend mutton and pork at high sensitivity. The mitochondrial 16S rRNA genes have been used as molecular markers to identify mammals, birds, shrimp, and other species using species-specific primers that amplify the 12S rRNA or 16S rRNA gene regions from mtDNA[17,22]. Gene loci on the mitochondrial genome have been used in species identification. These include the 12S and 16S rRNA loci. The D-loop (displacement loop) has been used less in species identification but more in intraspecies identification. Due to the greater sequence variation at this non-coding locus, it is now being used as a tool for identifying the presence of particular species within mixture of many species [23,24].

The secondary and confirmatory assay for specificity of primer pairs used in study is sequences of PCR products. Eight amplicons from each were sent for sequencing using Sanger technique (Macrogen/Korea). The retrieved sequences firstly must be trimmed to remove unwanted sequences before submitting them for BLASTN. The trimming performed by Bioedit to get the finally processed sequences. Abbreviation of *homo sapiens* sequences were used as (HIS-1 to HIS-8), *Ovis aries* sequences be (IOA-1 to IOA-8), *Capra hircus* sequences be (IBCH-1 to IBCH-8) and *Bos taurus* sequences be (IBT-1 to IBT-8).

The identity percentage and alignment results of amplified 16S rRNA gene of *homo sapiens*, *Ovis aries*, *Capra hircus* and *Bos taurus* with database were illustrated in table (3,4,5,6) respectively.

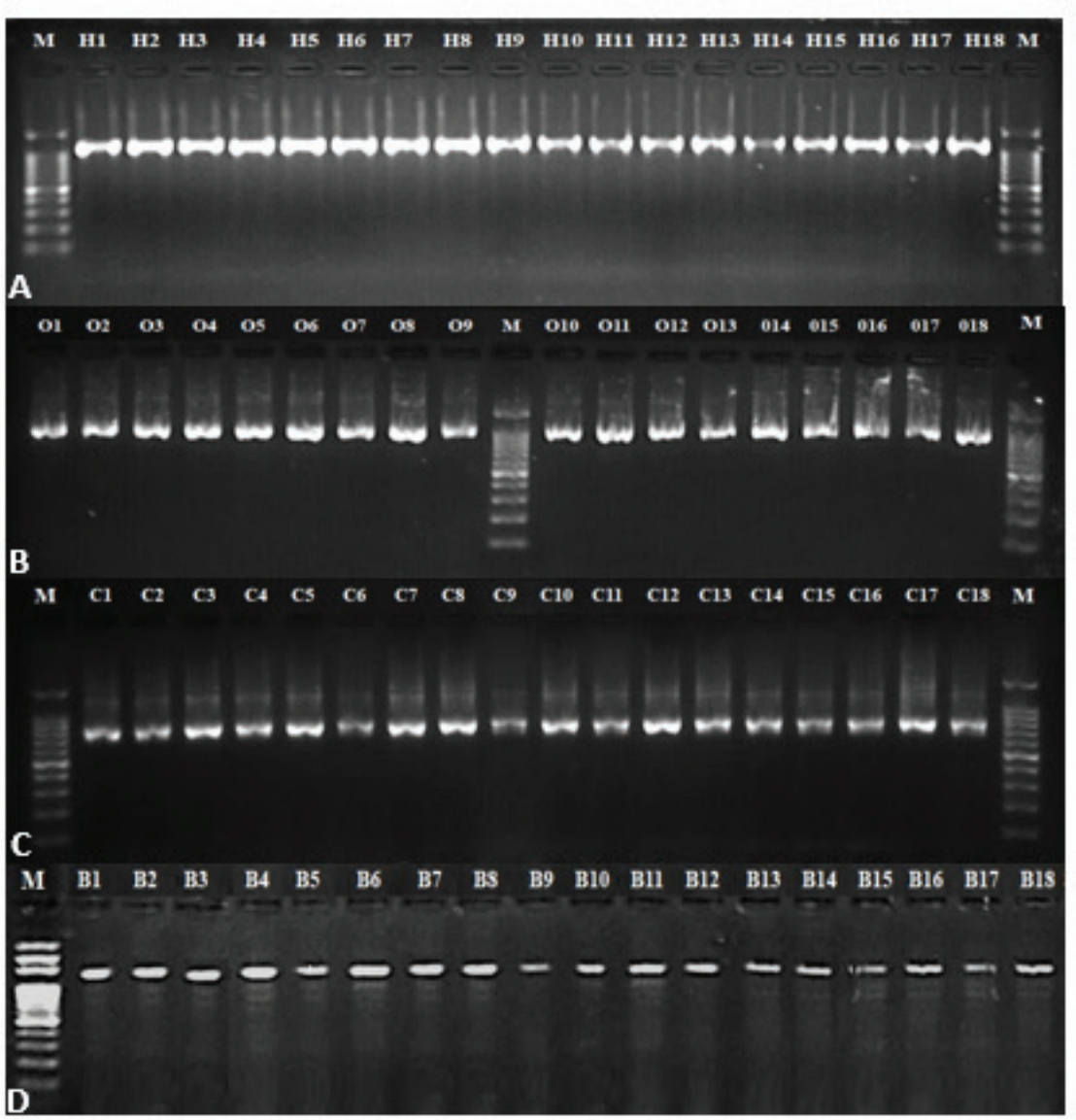

Figure 1: Agarose gel electrophoresis 1.5% for: A: 1200bp amplicon of *Homo sapiens* **16S rRNA gene. lane H1-H18 represent samples. B: 1060bp amplicon of** *Ovis aries* **16S rRNA gene. lane H1-H18 represent samples. C: 820bp amplicon of** *Capra hircus* **16S rRNA gene. lane H1-H18**

Table (3): Identity of blasted isolates (IHS-1 to IHS-8) with reference sequences of highest identity percentage

Isolate	Sequence ID	Expect	Identities	Gaps	Strand
IHS-1	MH444415.1	0.0	98.98%	0/885(0%)	Plus/Plus
IHS-2	MK069579.1	0.0	99.74%	0/771(0%)	Plus/Plus
IHS-3	MK069579.1	0.0	99.57%	2/697(0%)	Plus/Plus
IHS-4	MK069579.1	0.0	99.08%	0/654(0%)	Plus/Plus
IHS-5	MK059695.1	0.0	99.86%	0/701(0%)	Plus/Plus
IHS-6	MK069579.1	0.0	99.39%	0/657(0%)	Plus/Plus
IHS-7	MK295855.1	0.0	99.50%	0/599(0%)	Plus/Plus
IHS-8	MK069579.1	0.0	99.69%	0/653(0%)	Plus/Plus

Table (4): Identity of blasted isolates (IOA-1 to IOA-8) with reference sequences of highest identity percentage

Isolate	Sequence ID	Expect	Identities	Gaps	Strand
IOA-1	MG489885.1	0.0	98.70%	0/769(0%)	Plus/Plus
IOA-2	MG489885.1	4e-170	100.00%	0/329(0%)	Plus/Plus
IOA-3	MG489885.1	0.0	98.98%	0/586(0%)	Plus/Plus
IOA-4	MG489885.1	0.0	99.73%	0/749(0%)	Plus/Plus
IOA-5	MG489885.1	0.0	99.63%	0/542(0%)	Plus/Plus
IOA-6	MG489885.1	2e-157	99.36%	0/312(0%)	Plus/Plus
IOA-7	MG489885.1	0.0	99.17%	2/483(0%)	Plus/Plus
IOA-8	MG489885.1	0.0	99.80%	0/489(0%)	Plus/Plus

Table (5): Identity of blasted isolates (IBCH-1 to IBCH-8) with reference sequences of highest identity percentage

Isolate	Sequence ID	Expect	Identities	Gaps	Strand
IBCH-1	KP271023.1	0.0	99.02%	0/614(0%)	Plus/Plus
IBCH-2	KP271023.1	0.0	98.58%	0/633(0%)	Plus/Plus
IBCH-3	KP271023.1	2e-174	99.74%	0/378(0%)	Plus/Plus
IBCH-4	KP271023.1	0.0	100.00%	0/729(0%)	Plus/Plus
IBCH-5	KP271023.1	0.0	99.83%	0/595(0%)	Plus/Plus
IBCH-6	KP271023.1	0.0	100.00%	0/480(0%)	Plus/Plus
IBCH-7	KP271023.1	0.0	99.84%	0/618(0%)	Plus/Plus
IBCH-8	KP271023.1	0.0	99.17%	0/481(0%)	Plus/Plus

Table (6): Identity of blasted isolates (IBT-1 to IBT-8) with reference sequences of highest identity percentage

Isolate	Sequence ID	Expect	Identities	Gaps	Strand
IBT-1	MF169214.1	0.0	99.50%	3/601(0%)	Plus/Plus
IBT-2	KT184455.1	7e-139	100.00%	0/273(0%)	Plus/Plus
IBT-3	KT184466.1	0.0	99.32%	7/1177(0%)	Plus/Plus
IBT-4	KT184466.1	0.0	99.90%	0/979(0%)	Plus/Plus
IBT-5	KT184466.1	0.0	99.90%	0/965(0%)	Plus/Plus
IBT-6	KT184466.1	0.0	100.00%	0/512(0%)	Plus/Plus
IBT-7	KT184466.1	0.0	99.81%	1/1077(0%)	Plus/Plus
IBT-8	KT184466.1	0.0	100.00%	0/1092(0%)	Plus/Plus

The sequencing of the 16S rRNA has revolutionized the study and identification of human and non-human in forensic. Many study development a simple method using universal primers for species identification based on direct PCR sequencing using primer sets were designed based on the conserved regions of the 16S rRNA loci detected by the comprehensive sequence comparison among 30 animals whole[25]. Mitochondrial DNA the method could be a dominant tool for mammalian species identification, especially in forensic cases in which many unidentified biological samples must be analyzed such as blood spots[25,]. The 16S and 12S sequences

allowed identification of most species to the genus level Faster-evolving DNA regions are required to identify closely-related animals species[26]. the successfully used forensically informative nucleotide sequencing analysis of the 16S rRNA mitochondrial DNA to identify before unknown biological specimens of human and animals[27]. The mitochondrial 12S rRNA and 16S rRNA genes, including those from fish and amphibians to mammals including human beings. Therefore, universal primers were designed to amplify sequences in the fast-evolving animal mtDNA[17]. the PCR amplifications of mitochondrial 16S rRNA followed by sequencing and analysis showed to be very efficient for identification of species origin of species[21]. The 12S rRNA and 16S rRNA gene sequences of animals reveal the fitting level of interspecific variation but the great level of intraspecific homogeneity[28].

The results showed no cross-reactivity of designed primer pairs and the PCR assay based on the designed primer pairs will be simple, fast, sensitive, specific, and cost-effective.

Registration of Sequences in GenBank:

All the 32 sequence of 16S rRNA gene were submitted to GenBank for registration. After checking and revision the following accession numbers were donated:

16S rRNA *homo sapiens* (Human): MN192057, MN192058, MN192059, MN192060, MN192061, MN192062, MN192063, MN192064(Appendix 4-69 to 4-76).

16S rRNA *Ovis aires* (Sheep):MN173528, MN173529, MN173530, MN173531, MN173532, MN173533, MN173534, MN173535(Appendix 4-77 to 4-84).

16S rRNA *Capra hircus* (Goat): MN173285, MN173286, MN173287, MN173288, MN173289, MN173290, MN173291, MN173292(Appendix 4-85 to 4-92).

16S rRNA *Bos taurus* (Cow): MN197611, MN197612, MN197613, MN197614, MN197615, MN197616, MN197617, MN197618 (Appendix 4-93 to 4-100).

Conclusion

Sensitivity, specificity and accuracy of the designed species specific primer pairs and applicability of the designed primer pairs in forensics to investigate blood sports or evidence belonging for human, sheep, goat and cow.

Ethical Clearance: The project plan displayed on the scientific committee and scientific ethical committee of the department of Biology-college of science at university of Babylon and get approval

Source of Funding: There is no funding source and it is completely covered by authors

Conflict of Interest: There is no conflict of interest

References

1. D'Souza AR, Minczuk M. Mitochondrial transcription and translation: overview. Essays in biochemistry. 2018 Jul 20;62(3):309-20.
2. Herbers E, Kekäläinen NJ, Hangas A, Pohjoismäki JL, Goffart S. Tissue specific differences in mitochondrial DNA maintenance and expression. Mitochondrion. 2019 Jan 1;44:85-92.
3. Indo HP, Suenaga S, Tomita K, Ito H, Matsui H, Majima HJ. Analysis of oxidative stress marker, mitochondrial DNA copy numbers and mitochondrial DNA oxidation among 135 persons who live in Amami islands, a high centenarian population district in Kagoshima. Free Radical Biology and Medicine. 2018 May 20;120:S134.
4. Amorim A, Fernandes T, Taveira N. Mitochondrial DNA in human identification: a review. PeerJ Preprints. 2019 Jan 24;7:e27500v1.
5. Lee WC, Lin CS, Ko FC, Cheng W, Lee MH, Wei YH. Low mitochondrial DNA copy number of resected cecum appendix correlates with high severity of acute appendicitis. Journal of the Formosan Medical Association. 2019 Jan 1;118(1):406-13.
6. Mitra I, Roy S, Haque I. Application of molecular markers in wildlife DNA forensic investigations. Journal of Forensic Science and Medicine. 2018 Jul 1;4(3):156.
7. Mahmoodi M, Afshari KP, Seyedabadi HR, Aboozari M. Sequence analysis of 12S rRNA and 16S rRNA mitochondrial genes in Iranian Afshari sheep. Banat's Journal of Biotechnology. 2018 Jul 1;9(18).

8. Andrejevic M, Markovic MK, Bursac B, Mihajlovic M, Tanasic V, Kecmanovic M, Keckarevic D. Identification of a broad spectrum of mammalian and avian species using the short fragment of the mitochondrially encoded cytochrome b gene. Forensic Science, Medicine and Pathology. 2019 Jun 14;15(2):169-77.
9. Horreo JL, Fitze PS, Jiménez-Valverde A, Noriega JA, Pelaez ML. Amplification of 16S rDNA reveals important fish mislabeling in Madrid restaurants. Food control. 2019 Feb 1;96:146-50.
10. Yan L, She Y, Elzo MA, Zhang C, Fang X, Chen H. Exploring genetic diversity and phylogenic relationships of Chinese cattle using gene mtDNA 16S rRNA. Archives Animal Breeding. 2019 Jun 12;62(1):325-33.
11. Lavrov DV, Pett W. Animal mitochondrial DNA as we do not know it: mt-genome organization and evolution in nonbilaterian lineages. Genome biology and evolution. 2016 Aug 24;8(9):2896-913.
12. Untergrasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. Primer3–new capabilities and interfaces. Nucleic Acids Research.
13. Ahmed NS, Hadi YA, Dhefer IH. Polymorphism Study of TCF7L2 gene and related to some biochemical parameters in DM2 females Iraqi patients. Research Journal of Science and Technology. 2019 Jan;11(1):01-8.
14. Jarrar YB, Ghishan M. The Nudix Hydrolase 15 (NUDT15) Gene Variants among Jordanian Arab Population. Asian Pacific journal of cancer prevention: APJCP. 2019 Mar 26;20(3):801-8.
15. Cseke LJ, Kirakosyan A, Kaufman PB, Westfall MV. Handbook of molecular and cellular methods in biology and medicine. CRC press; 2016 Apr 19.
16. Heuvel JP. PCR Protocols in Molecular Toxicology. CRC Press; 2019 Jul 17.
17. ang L, Tan Z, Wang D, Xue L, Guan MX, Huang T, Li R. Species identification through mitochondrial rRNA genetic analysis. Scientific reports. 2014 Feb 13;4:4089.
18. Luo S, Valencia CA, Zhang J, Lee NC, Slone J, Gui B, Wang X, Li Z, Dell S, Brown J, Chen SM. Biparental inheritance of mitochondrial DNA in humans. Proceedings of the National Academy of Sciences. 2018 Dec 18;115(51):13039-44.
19. Barshad G, Marom S, Cohen T, Mishmar D. Mitochondrial DNA transcription and its regulation: an evolutionary perspective. Trends in Genetics. 2018 Sep 1;34(9):682-92.
20. Sharifi RS, Sofla SS, Seyedabadi HR. Genetic Diversity and Molecular Phylogeny of Iranian Goats Based on Cytochrome Oxidase I (COXI) Gene Sequences (KERAGAMAN GENETIK DAN FILOGENI MOLEKULER KAMBING-KAMBING IRAN BERDASARKAN SEKUENS GEN CYTOCHROME OXIDASE I (COXI)). Jurnal Veteriner. 2018;18(4):565-70.
21. Mane BG, Mendiratta SK, Tiwari AK, Narayan R. Sequence analysis of mitochondrial 16S rRNA gene to identify meat species. Journal of applied animal research. 2013 Mar 1;41(1):77-81.
22. Xu J, Zhao W, Zhu M, Wen Y, Xie T, He X, Zhang Y, Cao S, Niu L, Zhang H, Zhong T. Molecular identification of adulteration in mutton based on mitochondrial 16S rRNA gene. Mitochondrial DNA Part A. 2016 Jan 2;27(1):628-32.
23. Mitani T, Akane A, Tokiyasu T, Yoshimura S, Okii Y, Yoshida M. Identification of animal species using the partial sequences in the mitochondrial 16S rRNA gene. Legal medicine. 2009 Apr 1;11:S449-50.
24. Linacre A, Tobe SS. An overview to the investigative approach to species testing in wildlife forensic science. Investigative genetics. 2011 Dec;2(1):2.
25. Kitano T, Umetsu K, Tian W, Osawa M. Two universal primer sets for species identification among vertebrates. International journal of legal medicine. 2007 Sep 1;121(5):423-7.
26. Cawthorn DM, Steinman HA, Witthuhn RC. Evaluation of the 16S and 12S rRNA genes as universal markers for the identification of commercial fish species in South Africa. Gene. 2012 Jan 1;491(1):40-8.
27. Guha S, Goyal SP, Kashyap VK. Genomic variation in the mitochondrially encoded cytochrome b (MT-CYB) and 16S rRNA (MT-RNR2) genes: characterization of eight endangered Pecoran species. Animal genetics. 2006 Jun;37(3):262-5.
28. Mahmoodi M, Afshari KP, Seyedabadi HR, Aboozari M. Sequence analysis of 12S rRNA and 16S rRNA mitochondrial genes in Iranian Afshari sheep. Banat's Journal of Biotechnology. 2018 Jul 1;9(18).