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MOLECULAR DETECTION OF RAW MEAT FOR SOME ANIMAL SPECIES USING RFLP-PCR TECHNIQUE

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ABSTRACT

Mitochondrial cytochrome b gene plays a serious role in studying adulteration of meat species. This research study designed to distinguish the raw meat species of sheep, goat, cattle and donkey using RFLP-PCR technique of a universal cyt b gene 359bp. Ten indigenous samples were collected from each animal in different parts of the body. All the samples were processed for DNA isolation and amplified with a Polymerase chain reaction (PCR). The amplicons were cleavage with HinfI and RsaI restriction enzymes, digestion of PCR product resulted in production of specific characterization bands for each species then analyzed by agarose electrophoresis. *HinfI* RE created three fragments for sheep, goat and cattle, with some similarities in a few bands between them, while yielded two bands for donkey. *RsaI* RE produced two bands for all species with different length except sheep and goats have the same length. Thus, results recommend that the RFLP-PCR technique with *HinfI* and *RsaI* play an important role to detect the animal meat species, since it is a fast, simple and easily handle method for identification of animal species.

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INTRODUCTION

Several sources of meat products in different species mixed together to make a new inexpensive product and unnoticeable by physical testing (Ong *et al*., 2007). Regrettably, in many countries (specifically development countries) meat adulteration has always been a concern for several reasons such as health, economic and religious concerns (Khan *et al.*, 2018). Thus, detection of the origin species existing in meat products has become vital to keep people from illegal and undesirable corruption (Ghovvati *et al*., 2009, Ciupa *et al*., 2012). Nowadays, many identification techniques have been used for determination of species origin make up in raw meat like sensory analysis, anatomical (Arcos-Garcia *et al*., 2002) histological and immunological separation, although these methods have constraints (Ilhak and Arsalan, 2007), the main issues such as complexity, effort, specificity, costly and insufficiency to separate closely related species (Abdel-Rahman *et al*., 2009).

Various molecular methodologies have been made to differentiate origin species from meat. These strategies can diminish the inadequacies of normal techniques. These molecular procedures incorporate PCR, RAPD, AFLP, DNA hybridization and RFLP (Arslan *et al*., 2005; Dilger *et al*., 2020). Polymerase chain reaction methods are exceptionally quick and dependable, and now they have turned

out to be a mainstream for meat detection in meat industry (Kesmen *et al*., 2010 and Khan *et al*., 2018). Especially the mitochondrial DNA (mtDNA) has been the most generally studied region of eukaryotic genomes which has played a vital role for being developed of citizens and heredities. The mitochondrial cytochrome b gene (mt cyt b) encodes a famous protein that creates complex III of the mitochondrial phosphorylation system and is best single known encoded by the mitochondrial genome. This gene has been used widely as a vital utility tool in investigations of legal medicine and molecular progression (Prusak *et al*., 2004). This gene has been completely sequenced or partially sequenced for many species of Animalia family (Bravi *et al*., 2004 and Farag *et al*., 2015).

The study for detection of species origin depended on the mt cyt b gene have ranged between less than 400bp to more than 900bp RFLP-PCR DNA sequencing and variable size species specific multiplex PCR (Lee *et al*., 2009). A replacement DNA identification method is depended on the PCR amplification of a piece of the mt cyt b gene then digestion the amplicons by restriction enzymes via RFLP analysis, which produce species specific pattern, this technique requires less equipment and cost than sequencing (Ahmed, 2007; Abdel-Rahman *et al*., 2009 and Taha *et al*., 2021). Therefore, the objective of this study was to use RFLP-PCR analysis of the mt cyt b gene, using 2 restriction enzymes (HinfI and Rsa I) for differentiation of sheep, goat, cattle and donkey species in Erbil Kurdistan region.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

The present study was carried out during July 2019 to March 2020, on four local animals (Cattle, Goat, Sheep and Donkey) in both molecular genetics laboratory in college of agricultural engineering sciences Salahaddin University-Erbil-Iraq, and a private laboratory. The Meat samples were collected from slaughter houses in Erbil governorate and Donkey samples in some villages. Ten indigenous samples were collected from each animal in different parts of the body (mixed together to make poled samples).

Meat samples of the concerned species were stored in aluminum foil at -20 °C for DNA extraction. A commercial kit (Blood-Animal-Plant DNA Preparation), (Jena Bioscience GmbH, Germany) was employed to extract DNA from samples based on instructions of the manufactures. The obtained DNA was categorized and kept at -20 °C for the downstream applications. The quantity and quality of DNA was measured by Nanodrop spectrophotometer (UK) and gel electrophoresis. Purity of DNA ratio ranged between 1.7 and 1.9.

PCR Primers

Polymerase chain Reaction was arisen operating a modification of RFLP methods. The sequences of the used primer showed in Table 1.

Table (1): Sequence of cyt b primers used for detection of animal raw meat

Gene name	Nucleotide Sequences	amplified size	Reference
Cyt b (NP_904340.1, gene ID: Gene ID: 17711)	F: 5'CCATCCAACATCTCAGCATGATGA AA-3'	359 bp	Khan *et al* (2018)
	R:5'GCCCCTCAGAATGATATTTGTCCTCA-3'		

PCR Amplification

The pair of primers of the mt Cyt b gene for the species was run by PCR (Applied Biosystems® Veriti® 96-Well Fast Thermal Cycler, USA). The total volume of each reaction sample separately was 25µl, which is shown in Table 2. The conditions of the PCR program comprised of an initial denaturation at 95°C for 5 min, tracked by 35 cycles containing of denaturation at 95°C for 0.30 min, annealing 50°C 0.45 min, and then extension at 72°C for 45 sec, with final extension 72°C for 7 min. The products were run in a 2% agarose gel staining by ethidium bromide (LOT:110802BB197, Bio Basic Inc.) in Tris-borate EDTA buffer and pictured under UV Transilluminator (Biostep-UST-20M-8K).

Table (2): PCR reaction mixture for amplification of cyt b gene

PCR mixture	Required concentration	Amount µl
DNA template	50ng	5 µl
AMPLICON red Master Mix (2 X)	1X	12.5 µl
Pair Primers (20pmol/µl for each F,R)	20 pmol F and R	2 µl
DNAs free water	-	5.5 µl
Final reaction volume		25 µl

RFLP-PCR Analysis

The PCR products were assimilated with HinfI and RsaI (ADR6201 00001211493, Promega- USA) restriction enzymes for each sample separately. The mixture consisted (10X Reaction buffer 2 µl, Reaction Enzyme 5 U, PCR product 10 µl and filled with 7.5 µl of DNAes free water to complete 20 µl), then incubated for 2-4 hours at (37 °C) based to the instruction of the manufacture. Eight µl were electrophoresed thru 2.5% agarose gel. The lengths of the bands were compared with the 100 bp of Ladder RTU (Cat NO. DM012-R500, Promega- USA).

RESULTS AND DISCUSIONS

Genomic DNA was extracted and the purity of DNA ratio ranged between 1.7 and 1.9. The mt cyt b gene (NP_904340.1, gene ID: Gene ID: 17711) was successfully amplified 359 bp clearly as shown in Figure 1. Following the amplicons were faced to HinfI and Rsa I restriction enzymes, both enzymes cleaved the target DNA in different places. Then, digested products were run on 3% agarose gel electrophoresis. Each sample showed different fragment sizes after digestion with HinfI and Rsa I restriction endonucleases, when they were visualized under UV Transilluminator and identified by comparison of the standard size marker, as in Table 3 and Figure 2, 3, respectively.

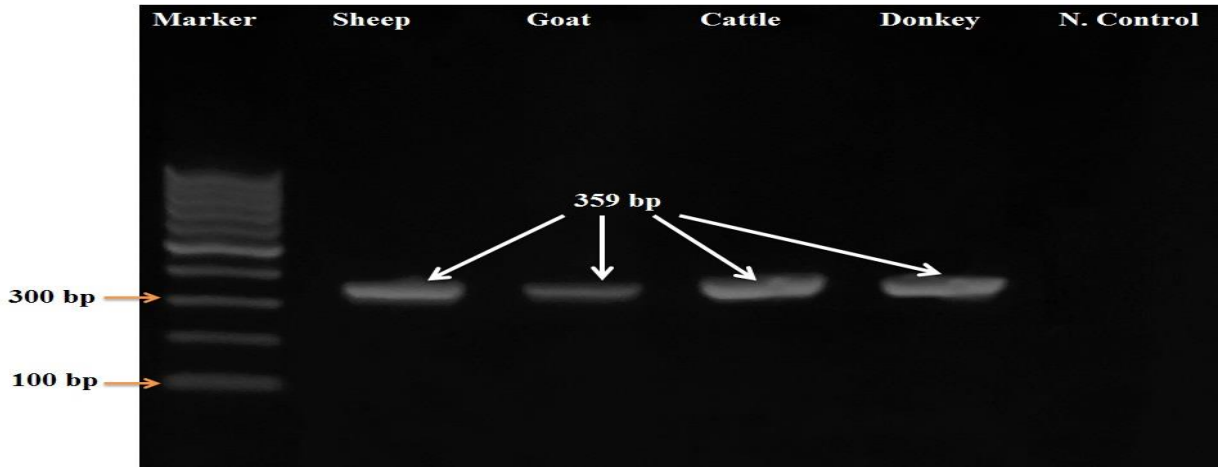

Figure (1): PCR product of four species, Sheep, Goat, Cattle and Donkey of Cyt B gene fragments were separated by 2% agarose gel electrophoresis (1.5 hours 80V 1X TBE buffer) stained with 5µl ethidium bromide.

Table (3): Number of bands and their sizes of animal species (sheep, goat, cattle and donkey) produced by digestion with (HinfI, RsaI) restriction enzymes.

Animal species	***HinfI* fragment size pb**	***Rsa I* fragment size pb**
Sheep	3 bands (196, 100,63)	2 bands (263, 96)
Goat	3 bands (196, 100,63)	2 bands (263, 96)
Cattle	3 bands (196, 110,53)	2 bands (311,48)
Donkey	2 bands (254,105)	2 bands (198, 161)

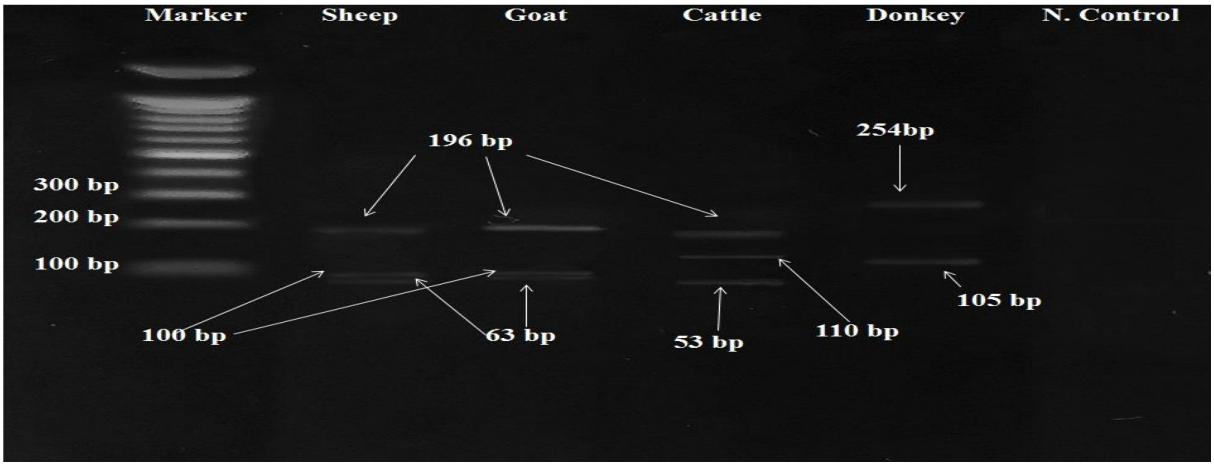

Figure (2): Digestion of PCR product of four species, Sheep, Goat, Cattle and Donkey with HinfI restriction enzyme of Cyt B gene fragments were separated by 2.5% agarose gel electrophoresis (1.5 hours 80V 1X TBE buffer) stained with 5µl ethidium bromide.

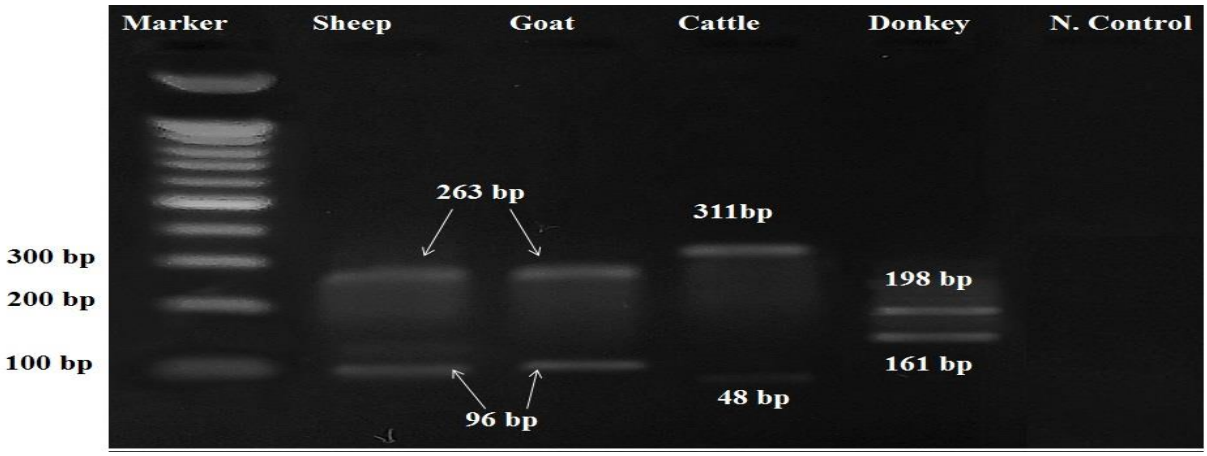

Figure (3): Digestion of PCR product of four species, Sheep, Goat, Cattle and Donkey with Rsa I restriction enzyme of Cyt B gene fragments were separated by 2.5% agarose gel electrophoresis (1.5 hours 80V 1X TBE buffer) stained with 5µl ethidium bromide.

Detection of meat and their products based on DNA identification methods always provide better determination and positive result compared with the other methods such as traditional morphological or protein identification, therefore DNA based methods are the most valuable technique for detecting origin species in commercial foods and animal products (Ali *et al*., 2015; Andrea *et al*., 2015). In this century, adulteration or deception in meat has become a common, so that it is critical getting confirmation on the meat authentication to discover the species that is an origin in such products, since it is a vital for food safety, consumer demands, countries 'laws and …etc., therefore seeking for precise identification of meat origin has become a vital element in food quality control procedures especially in poor countries (Farag *et al*., 2015 and Khan *et al*., 2018). In this study mt cyt b gene 359bp was used to identification and investigation of origin species of animal meat such as (sheep, goat, cattle and donkey) since this short mitochondrial fragment is repeated the widest range in nucleotide databases (Guo *et al*., 2005). Amplification of the pair primers of mt cyt b gene by PCR produced 359bp in all extracted DNA animal samples this confirms the more benefits of using high copy number mtDNA over nuclear DNA, which is approved by previous study (Khan *et al*., 2018; Dilger *et al*., 2020 and Taha *et al*., 2021).

So that, based on the number of fragments of RFLP technique that were obtained in the four animal species by using HinfI and Rsa I restriction enzymes, the digested result provided different restriction sites which differentiate the animal meat samples of origin species. Meat of two more species is more likely to be mixed while being processed in grinders, choppers. Therefore, all the samples have to be uniform and it has to take several samples of each species, since RFLP-PCR technique is more subtle and the best methods for meat species identification (Chikuni *et al*., 1990 and Khan *et al*., 2018). Analyzing the mt cyt b gene region by RFLP-PCR method revealed excellent power and effective, tool for differentiating all animal meat species samples such as (sheep, goat, cattle and donkey) because there were no any unspecific bands or fragments produced when digested with restriction enzymes among them. Thus, restriction enzymes presented precise bands for each sample and there was no need to use statistical analysis to detect significant or insignificant among the

samples, therefore the result was in agreement with that previously done by (Apostolidis *et al*.,2000). Moreover, the present results were partially in agreement with the results obtained by (Kušec *et al*., 2016 and Khan *et al*., 2018). Furthermore Bravi and coworkers (2004) revealed that the RFLP-PCR analyses with cyt b gene region showed high acceptance for identification animal species through the different restriction endonuclease. Whereas, the present results were partially agreement with the results obtained by (Kušec *et al*., 2016), and also it is a similar results with Nagata *et al.*, (2005) which used RFLP-PCR of cyt b gene to identification wild animal species by HnfI restriction enzyme.

CONCLUSIONS

Many biochemical techniques have been used for detection of origin meat species all over world. However, the present study was used the RFLP-PCR assay and indicated that this method was developed targeting mitochondrial cyt b gene for the differentiation of animal species by using HinfI and Rsa I restriction endonucleases and a reliable method to use widely in meat identification. It can also be counted as a fast 'since does not need statistical analysis', inexpensive and not laborer technique for finding a source of origin meat species in a forensic method. Which are equally valid and effective methods in many aspects in animal production such as breeding, protection of biodiversity and today's subject is adulteration of meat products and other human foods in many around the world. Moreover, it also can be used for authentication of fraudulent substitutions of low-price meat and defense the customer's right.

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CONFLICT TO INTEREST

The authors declare that they have no conflicts of interest.

الكشف الجزيئي للحوم الخام لبعض انواع الحيوانات باستخدام تقنية تباين اطوال قطع التقييد – تفاعل البلمرة المتسلسل

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الخلاصة

يلعب الموروث السيتوكرومي ب في الميتوكوندريا دورا مهما في دراسة انواع اللحوم المغشوشة. صممت هذه الدراسة البحثية لتمييز انواع اللحوم الخام من الاغنام والماعز والابقار والحمير باستخدام تقنية تباين اطوال قطع التقييد– تفاعل البلمرة المتسلسل من البادئ المعروف عالميا 359 قاعدة زوجية. تم جمع عشر عينات اصيلة من كل حيوان في اجزاء مختلفة من الجسم. وقد تم عزل الحمض النووي الدنا من جميع العينات وتضخمه باستخدام تقنية التفاعل البلمرة المتسلسل. ثم تم هضم القطع المضخمة مع الأنزيمات القاطعة Hinf1 وRsaI, وقد ادى هذا الهضم الى انتاج مجموعات توصيف محددة لكل نوع، ثم تم تحليل القطع المضخمة التي تم انتاجها عن طريق تباين أطوال قطع التقييد بواسطة الترحيل الكهربائي بالهلام. حيث تم

انتاج ثلاثة قطع من الأغنام والماعز والأبقار، مع بعض اوجه التشابه بينها في بضعة القطعات، في حين تم انتاج قطعتين للحمير بوسطة انزيم القطع Hinf1. وقد أنتج هذا الهضم بانزيم القطع RsaI قطعتين لجميع الانواع بحجم مختلف عدا الاغنام والماعز التي لها نفس الحجم. وتوصي النتائج بأن استخدام تقنية تباين أطوال قطع التقييد– تفاعل البلمرة المتسلسل مع إنزيمات القاطعة Hinf1 وRsaI, لها دورا هاما في الكشف عن انواع اللحوم الحيوانات لأنها طريقة سريعة وبسيطة وسهلة الاستخدام للتعرف على أصل ونوع الحيوانات.

الكلمات المفتاحية: التضخيم، الهضم، الموروث السيتوكرومي ب في الميتوكوندريا ، الأنزيمات القاطعة ، تحديد الأنواع

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