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The determination of meat species by PCR-RFLP method using mitochondrial ND4 gene in pastırma, a traditional dry cured meat product

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Abstract: Pastırma is a high value, traditional, dry cured, and edible coated meat product. For economic and sociocultural reasons, it is important to know which meat species are used in pastırma. The present study aims to carry out the determination of the meat species in pastırma. A total of 144 pastırma samples were collected from different stores. After genomic DNA isolation, the total DNAs obtained were subjected to polymerase chain reaction (PCR) using specially designed novel primers amplifying the mitochondrial *ND4* (*MT-ND4*) gene region, specific for cattle (*Bos taurus*), water buffalo (*Bubalus bubalis*), horse (*Equus caballus*), and donkey (*Equus asinus*) species. For the identification of the meat species, gel purified 952 bp PCR products were digested with fast digest *Saq*AI restriction enzymes selected based on preliminary in silico analyses. The results of the present study revealed that all of the pastırma samples were made from cattle meat. Cross-reactions and false-positive identifications are serious problems in routine determination of the meat species in food control laboratories. It is important to perform fast, inexpensive determination with high sensitivity and specificity for the identification of meat species. This study has shown that meat species can be detected with high specificity in products such as pastırma, which uses large pieces of meat cuts.

Key words: Meat adulteration, consumer demand, sensitive detection

1. Introduction

Animal proteins are an essential part of a daily diet in terms of high digestibility and they contain sufficient and balanced essential amino acids [1,2]. Various methods such as salting and drying have been developed for the long-term preservation of meat since ancient times [3]. Meats are also cured and coated with various herbs and spices in order to extend their shelf life and gain characteristic flavor and aroma [4]. Pastırma, one of the products made in this way, is an edible coated, well-known and traditionally dry cured meat product, mostly consumed in the east Mediterranean region, especially in Turkey. Traditional pastırma is produced only in big whole meat cuts derived from a single animal (cattle or water buffalo) that vary between 1.2 and 4.8 kg [5]. There are potential risks such that different meat species including horse and donkey that are not preferred by the society or that are of low quality can be used in the production of pastırma. In many countries, it is common practice to use meat of different origins to reduce production costs [6–8].

Determination and avoidance of manipulative practices to protect consumers with simple and rapid detection methods are needed. This is of utmost significance for conforming to the national standards and for protecting consumer preference [6].

The PCR-RFLP method has been a fast, easy, reliable, and valuable tool for the identification of meats of different animal species in recent years and has come into prominence as a strong method, especially when using highly conserved mitochondrial DNA (mtDNA) as the target region. In this manner, the sequence diversity gained with the PCR-RFLP method could be used to create a fingerprint for the identification of the origin of meat [7].

The studies on pastırma have generally focused on the product quality, the technology [9,10], and food safety [11,12]. However, there is a lack of studies aimed at evaluating pastırma in terms of the adulteration and the fraudulent substitution of meat according to the literature. The adulteration of meat-based products is a major problem in the meat industry due to the economic, religious, and cultural concerns of consumers. Increased

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consumer awareness of food safety and quality has led to the development of legal regulations by local authorities for the authentication of meat and meat products in food industries. Therefore, it is aimed to design fast, inexpensive, and convenient methods for determining the possible meat species used in pastırma production.

2. Materials and methods

2.1. Samples collection

A total of 144 pastırma samples were collected from different stores in Kayseri province of Turkey. For positive controls, cattle (*Bos taurus*), water buffalo (*Bubalus bubalis*), donkey (*Equus asinus*), and horse (*Equus caballus*) blood samples were collected from nearby farms and villages. Blood samples collected with EDTA vacuum tubes were subjected to gDNA extraction immediately.

2.2. Extraction of genomic DNA

After removal of the edible coat, total meat of 5 g from the pastırma samples was completely homogenized. The total DNA extraction was performed using the AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen, China) on the pastırma and blood samples according to the manufacturer's protocol. The concentrations of the gDNA samples (μg/μL) obtained were measured by Qubit 3.0 fluorometer (Thermo Fisher, USA). DNA samples were stored at –20 °C until they were analyzed.

2.3. Verification of gDNA samples as of mammalian origin with universal primers

Genomic DNA obtained from pastırma samples was confirmed as of mammalian origin by the PCR using the following primer pairs: forward 5'-CGAAGCTTGATATGAAAAACCATCGTTG-3' and reverse 5'-TGTAGTTRTCWGGGTCHCCTA-3' [13]. Maxima Hot Start Green 2X PCR Master Mix (Thermo Fisher, USA) was used for the PCR analysis according to the manufacturer's instructions. The PCR amplification was performed with an initial denaturation of 95 °C for 4 min followed by 30 cycles, each consisting of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 s. The final extension cycle was performed at 72 °C for 10 min (Arktic Thermal Cycler; Thermo Fisher, USA). The gels were stained with GelRed Nucleic Acid Gel Stain (Biotium, USA) and 772 bp PCR products were visualized under a UV transilluminator (Vilber Lourmat, France).

2.4. Primer design

A single universal primer that can amplify the mitochondrial NADH dehydrogenase subunit 4 (*MT-ND4*) genes from the cattle, water buffalo, horse, and donkey species was designed to determine the meat species of the samples. The properties of novel designed primers are summarized in Table 1. In order to evaluate the amplification of the *MT-ND4* gene of the selected animal species, different mitochondrion complete genomes in the NCBI database were selected randomly for each species. Previously described guidelines were followed to design primers [14]. The novel designed primer pairs were checked using the Primer-BLAST program to confirm the specificity and the absence of nonspecific amplification [15]. Designed novel primers binding regions of randomly selected GenBank accessions of cattle, water buffalo, horse, and donkey species are shown in Figure 1.

2.5. PCR-RFLP assay

Maxima Hot Start Green 2X PCR Master Mix (Thermo Fisher, USA) was used for PCR analysis according to the manufacturer's instructions. PCR amplification was performed with an initial denaturation of 95 °C for 4 min followed by 35 cycles, each consisting of 95 °C for 30 s, 42 °C for 30 s, and 72 °C for 1 s. The final extension cycle was performed at 72 °C for 10 min (Arktic Thermal Cycler; Thermo Fisher, USA). All amplification products were analyzed by agarose gel (1.5%) electrophoresis at 100 V for 45 min. The gels were stained with GelRed Nucleic Acid Gel Stain (Biotium, USA) and were visualized under a UV transilluminator (Vilber Lourmat, France). All 952 bp PCR products were purified using the GeneJET Gel Extraction Kit (Thermo Fisher, USA) according to the manufacturer's protocols.

All in silico analyses in the study were performed using Geneious Bioinformatics Software version R11 [16]. Commercially available Fast Digest *Saq*AI enzymes were selected as restriction endonucleases to produce discriminative RFLP patterns, and RFLP assay was performed on gel purified PCR products following the manufacturer's protocols (Thermo Fisher, USA). The summaries of in silico PCR-RFLP band patterns that generated digest enzyme are shown in Table 2.

Table 1. The properties of novel designed primers.

Primers	Target gene	Sequence (5' to 3')	Length (bp)	%GC	Products size (bp)
ND4-F	*MT-ND4*	GAAGCCACATTAGTTCCAACAC	22	45.5	952
ND4-R		GGAGGGCTATGAGTGCGTTT	20	55	

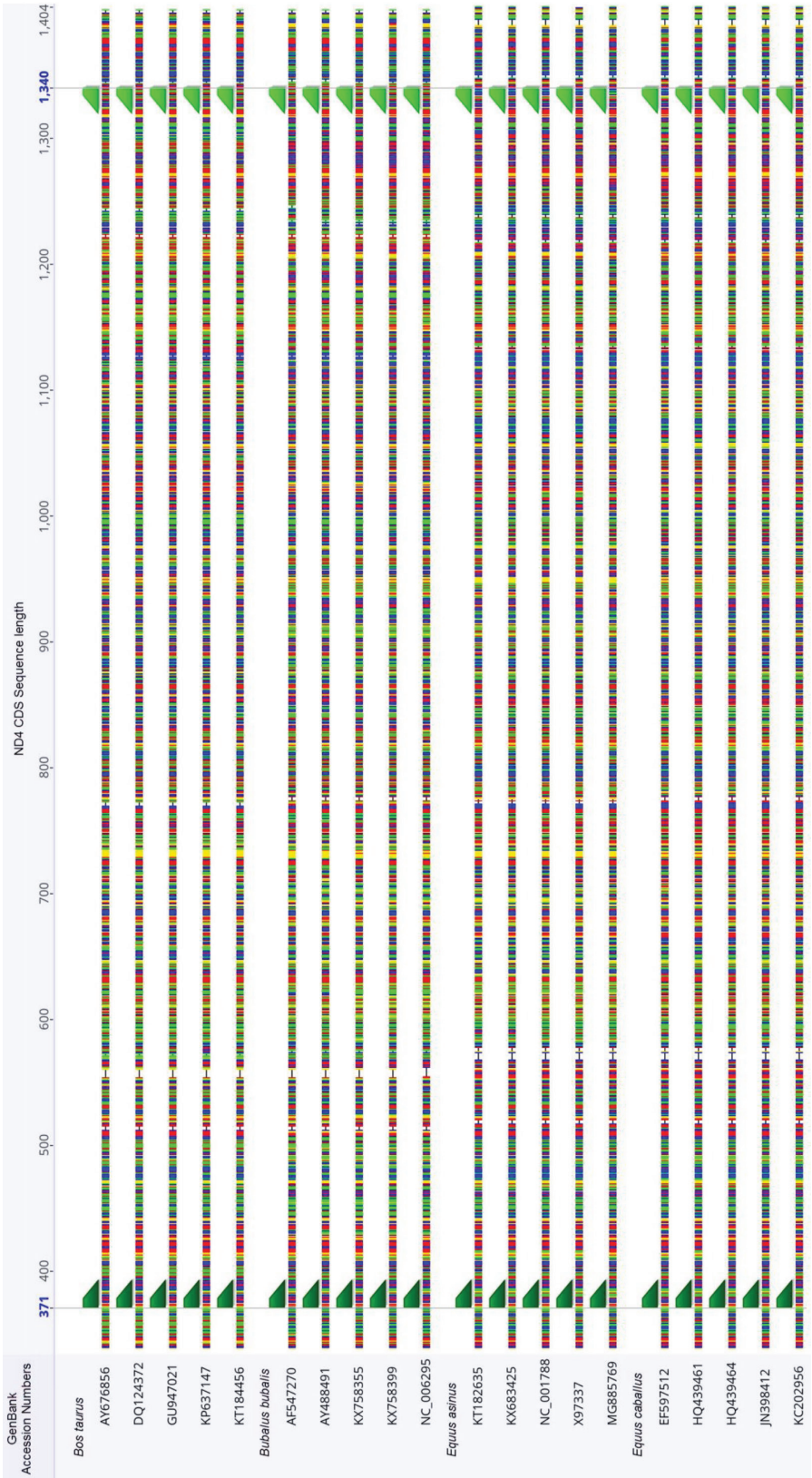

Figure 1. Designed novel primers binding sequences of randomly selected GenBank accession of *MT-ND4* CDS in mitochondrion complete genomes (Dark green primer ND4-F sequence: 371-392, Light green primer ND4-R sequence: 1320-1339).

3. Results

It was determined that mammalian gDNAs were successfully extracted from all pastırma and blood samples. Universal PCR results of some pastırma samples are given in Figure 2. Pastırma and blood samples were subjected to *MT-ND4* amplification with novel universal primers. The gel purified 952 bp PCR products (Figure 3) were digested with fast digest *Saq*AI enzyme and this allowed to differentiate 4 species that were studied. All of the 144 pastırma samples analyzed were found to be produced from cattle meat. Water buffalo, horse, and donkey meat were not detected in the samples. PCR-RFLP profiles of blood and pastırma samples are shown in Figure 4.

4. Discussion

In this study, it was aimed to determine the possible adulterations in pastırma, which is a traditional meat product in Turkey using trimmed large pieces of meats. For this purpose, a fast, sensitive, and novel PCR-RFLP method was developed by using a single primer pair and fast digest *Saq*AI enzyme. In this method, the *MT-ND4* gene was barcoded and cattle, water buffalo, horse, and donkey species were specifically identified. All of the 144 pastırma samples that were analyzed did not contain water buffalo, donkey, or horse meat. In the past, various animal species were investigated in meat and meat products. A study conducted by Di Pinto et al. [17] demonstrated a high substitution rate among the meat products, highlighting a mislabeling rate of 57%. Similarly, in a study performed in the United States, 10 out of 48 analyzed samples of ground meat were found to be mislabeled and contained additional meat species based on real time PCR [18]. In a study conducted by Ayaz et al. [19] in Turkey, 22% of the meat product samples were not in compliance with the Turkish food codex, thus violating the consumer rights and presenting a potential public health risk. Particularly, due to the horse meat scandal in 2013 in Europe, a number of methods have been developed to specifically address the adulteration of meat and meat products.

The primers used in the present study excellently amplified the animal species tested from NADH dehydrogenase subunit 4 (*MT-ND4*) gene fragments. PCR amplification produced a 952 bp fragment for each of these species. The amplicons were digested with the restriction

Table 2. The summary of in silico PCR-RFLP results that generated *Saq*AI fast digest enzyme (bp).

	Bos taurus (cattle)	*Bubalus bubalis* (water buffalo)	*Equus caballus* (horse)	*Equus asinus* (donkey)
*Saq*AI	759 147 50	372 342 104 98 44	588 168 161 41	506 249 201

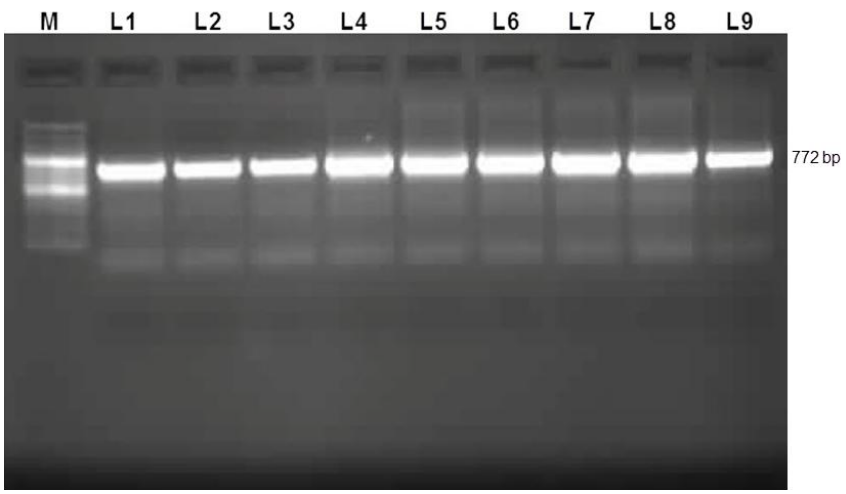

Figure 2. Agarose gel electrophoresis results of PCR with universal mammals specific primers. M: Molecular weight marker (Gene Ruler 100-bp DNA Ladder, Thermo), L1–9: Some mammalian positive isolates.

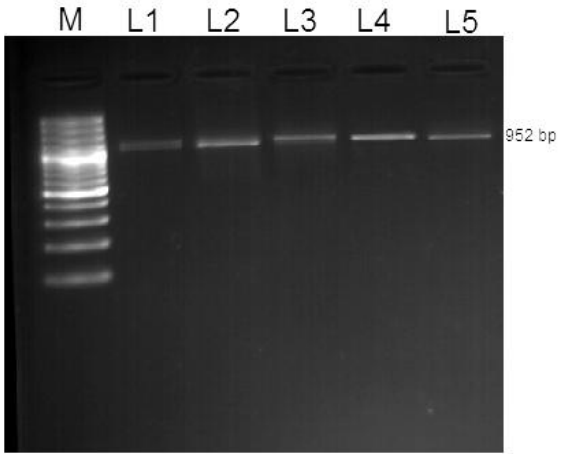

Figure 3. Agarose gel electrophoresis results of gel purified *MT-ND4* amplicons before RFLP with novel primers. M: Molecular weight marker (Gene Ruler 100-bp DNA Ladder, Thermo), L1: Positive control for cattle, L2: Positive control for water buffalo, L3: Positive control for donkey, L4: Positive control for horse, L5: Pastırma sample.

enzyme *Saq*AI and a pattern capable of identifying and distinguishing each of the above species was obtained. The RFLP pattern of restriction enzymes was highly specific for the meat samples. Similarly, Meyer et al. [20], Girish et al. [21], Haider et al. [5], and Ali et al. [22] amplified the cytochrome b gene using PCR followed by digestion enzymes to identify variable animal species' meat. In the context of all these studies, it can be seen that the PCR-RFLP method is a fast and easily applicable method, with high specificity for the authentication of meat and meat products.

The *Saq*AI restriction enzyme was very efficient to differentiate all of these species and was highly species-specific. This might be due to the high level of protection of the mtDNA sequence in animal species. To identify the meat and meat products that originated from different animal species, various methods were designed such as PCR [23], randomly amplified polymorphic DNAs (RAPDs) [24], amplified fragment length polymorphisms (AFLPs) [25], DNA hybridization [26], terminal-RFLP (T-RFLP) [7], DNA biochip [27], and many biomolecular methods. Moreover, DNA sequencing is expensive, time-consuming, and unsuitable for routine species' identification studies [21]. As well as molecular methods, serologic and morphological methods can also be used in animal species' identification in foods, but these conventional methods have not provided sensitive and accurate identification in processed foods. All of these methods have their own advantages, disadvantages, and self-limitations.

PCR-RFLP of the mitochondrial genes is highly repeatable, cheap, practical, and quicker than the methods stated above [5,20,28]. In addition, *MT-ND4* and fast digest *Saq*AI combinations could be more efficient as being low-cost and faster for routine identification of meat species. This procedure could be ideal for the identification of meat species because it does not require expensive nucleotide sequence and can be completed in less than 4 h including the whole experimental analysis and the evaluation of RFLP profiles.

5. Conclusion

According to the Turkish food codex, pastırma can only be produced from the meat of cattle or water buffalo. In spite of this, no water buffalo meat was found in the pastırma samples. Consumers have the right to know what they consume. For economic and religious reasons, the raw materials and the production process of animal origin foods

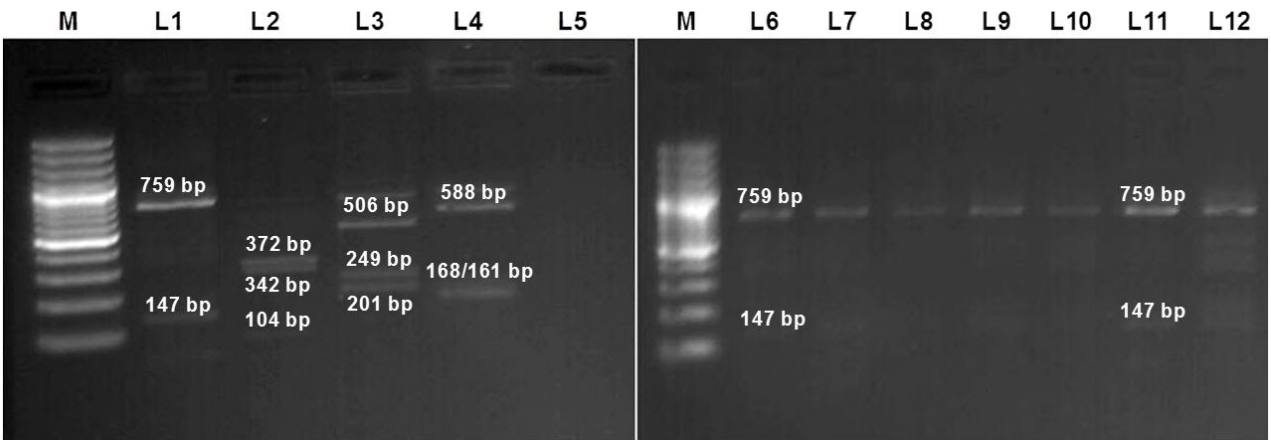

Figure 4. Agarose gel electrophoresis results of PCR-RFLP. M: Molecular weight marker (Gene Ruler 100-bp DNA Ladder, Thermo), L1: Positive control for cattle, L2: Positive control for water buffalo, L3: Positive control for donkey, L4: Positive control for horse, L5: no DNA, L6–12: Some gDNA samples of pastırma.

should be clearly stated. For this reason, it is very important to be able to sensitively detect the meat species used in the manufacturing of meat products. The DNA-based molecular analyses are recommended because of the disadvantages of cross-reactions or false positivity in serological tests. In the present study, designed PCR-RFLP protocols were found to be highly sensitive in determining the meat species that were possibly used in the pastırma production.

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