ORIGINAL ARTICLE

Identification of commercial meats from Amazonas, Peru using PCR-RFLP of mitochondrial 12S rRNA gene

Identificação de carnes comerciais do Amazonas, Peru, por PCR-RFLP do gene mitocondrial 12S rRNA

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Abstract

The use of analytical methodologies based on DNA (e.g. PCR-RFLP) to determine the authenticity of different types of meat products after the initial labeling is pivotal to avoid fraudulent practices due to the increased rates of meat consumption. Our PCR-RFLP of mitochondrial 12S rRNA gene using restriction enzymes (*AluI* and *ApoI*) aimed to confirm the accuracy of the meat species labeling, based on fresh and processed meat collected in central markets along the main cities in the Amazonas Region (Bagua, Bagua Grande, Chachapoyas, Luya, Pedro Ruiz, Rodriguez de Mendoza). Our analyses qualitatively identified and differentiated three commercial species of fresh meat (bovine, porcine, ovine) and also found the substitution of goat by sheep meat. Regarding processed meat, its composition was uncertain and further analyses should be addressed to determine the meat origin. Monitoring using DNA-based analytical methods of meat trade is suggested to determine fraudulent practices, such as species substitution in markets along regions of Peru.

Keywords: Amplicon; Enzymatic digestion; Mitochondrial DNA; Restriction fragments; Species substitution; Peru.

Resumo

O uso de metodologias analíticas baseadas em DNA (PCR-RFLP) para determinar, após a rotulagem inicial, a autenticidade de diferentes tipos de produtos derivados de carne é essencial para evitar práticas fraudulentas, tendo em vista o crescente aumento do consumo de carne. Nossa análise por PCR-RFLP do gene mitocondrial 12S rRNA usando enzimas de restrição (*AluI* e *ApoI*) teve como objetivo confirmar a precisão da rotulagem de espécies de carne animal, com base em amostras de carne fresca e processada, coletadas em mercados centrais nas principais cidades da região do Amazonas (Bagua, Bagua Grande, Chachapoyas, Luya, Pedro Ruiz, Rodriguez de Mendoza). Nossas análises identificaram e diferenciaram qualitativamente três espécies comerciais de carne fresca (bovina,

suína, ovina) e também encontraram a substituição de espécies de cabra por carne ovina. Em relação à carne processada, sua composição mostrou-se incerta e análises adicionais devem ser feitas para determinar a origem desta. Sugere-se o monitoramento do comércio de carne usando métodos analíticos baseados no DNA para determinar práticas fraudulentas como substituição de espécies nos mercados ao longo das regiões do Peru.

Palavras-chave: Amplificação; Digestão enzimática; DNA mitocondrial; Fragmentos de restrição; Substituição de espécies; Peru.

1 Introduction

The changes in consumer attitudes towards health and nutrition has generated a tremendous growth in meat consumption over the past several years (Rojas & García, 2007; Sumathi et al., 2015). This increased demand can promote adulteration/fraudulent practices that might include mislabeling, fraud, and substitution for other product (Sumathi et al., 2015). Species substitution is one of the most common mislabeling practice, in which low-value species is labeled as high-value (Sentandreu & Sentandreu, 2014; Spink et al., 2015).

Although some techniques, such as protein based methods, isoelectric focusing, liquid chromatography, and immunoassays, are available for meat species identification (Sumathi et al., 2015), they are not suitable in certain instances for routine sample analysis because proteins lose their biological activity after death, and their presence and characteristics depend on the cell types (Lockley & Bardsley, 2000). Approaches based on DNA to identify species is well established and widely used in food analysis because DNA offers advantages over proteins, due to its stability at high temperature, its presence in all tissue types, and greater variation with genetic code (Mackie, 1996; Asensio et al., 2002; Sumathi et al., 2015). These DNA molecular methods are highly specific, reliable, efficient and sensitive (Sawicki et al., 2006).

Among several techniques using DNA amplification for species identification, the most useful and advantageous is PCR-RFLP, which amplifies specific regions with universal PCR-primer system in combination with a few restriction enzymes (Sumathi et al., 2015; Ali et al., 2018). Currently, one of the most widely used specific regions for meat species identification have been based on the amplification of conserved mitochondrial DNA region (Sawicki et al., 2006; Ali et al., 2018), which can reveal genetic variation among species (Meyer et al., 1995; Yang et al., 2014).

The high rates of meat trade in central markets along the six main cities of the Amazonas Region, in northern Peru, possibly generated meat adulteration. Thereby, to confirm the accuracy of the meat species labeling in these markets, commercial meat was collected and, for the first time in Peru, meat species were further discriminated by PCR-RFLP of mitochondrial 12S rRNA gene.

2 Material and methods

2.1 Raw material

Fresh meat samples were collected in central markets of the main cities of the Amazonas Region in northern Peru: Bagua (5°34'51" S, 78°31'15" W), Bagua Grande (5°45'16" S, 8° 26'32" W), Chachapoyas (6°13'54'" S, 77°52'85" W), Luya (6°9'53" S, 77°56'40"W), Pedro Ruiz (5°56'39"S, 77°59'31"W), and Rodríguez de Mendoza (6°18'57" S, 77°32'17" W). Samples were transported and preserved at 4 °C. A total of 65 muscle tissue samples, including mincemeat (Table 1), were sampled to confirm the following meat species: bovine (*Bos taurus*), porcine (*Sus scrofa*), ovine (*Bovis aries*) and goat (*Capra aegagrus-hircus*). In addition, a total of nine processed meat samples (3 for bovine sausage, 3 for bovine ham, and 3 for regional hand-made sausage) were also analyzed to identify their species content.

Table 1. Number of fresh meat samples collected from different stores in the central markets of the main cities of the Amazonas Region.

Cities	Bovine	Porcine	Ovine	Goat	Mincemeat
Bagua	5	2	3	4	1
Bagua Grande	5	2	2	0	1
Chachapoyas	5	2	2	0	1
Luya	5	2	2	0	1
Rodriguez de Mendoza	5	2	2	0	1
Pedro Ruiz	5	2	2	0	1
Total	30	12	13	4	6

2.2 DNA extraction and amplification of mitochondrial 12S rRNA gene fragment

Genomic DNA was extracted from 50 mg of tissue using the Quick-DNA ™ Miniprep Plus Kit (Zymo Research, California, USA), following the manufacturer's instructions. The mitochondrial 12S rRNA gene was amplified using universal primers: 12S-rRNA-F and 12S-rRNA-R (Girish et al., 2005). This gene was amplified for fresh and processed meat samples using polymerase chain reaction (PCR) with MasterMix (Promega, Wisconsin, USA) in the following reaction mixture: 10 ng of DNA and 0.25-0.5 pmol of forward and reverse primers for a total volume of 10 µL and using a SimpliAmp thermal Cycler (Applied Biosystems, California, USA). The PCR protocol comprised an initial denaturing at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. Additionally, specific primer pairs (Table 2) to the mitochondrial 12S rRNA gene were used as internal controls (Aranguren-Méndez et al., 2009). The reaction mixture follows the reaction parameters above. The PCR protocol comprised an initial denaturing at 93 °C for 2 min, followed by 30 cycles of denaturation at 93 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 45 s, and final extension at 72 °C for 5 min. The PCR products were analyzed by electrophoresis in 1% agarose gel with SYBR-Green (ThermoFisher, Massachusetts, USA) staining.

Table 2. Universal and specific primers used for the amplification of the mitochondrial 12S rRNA gene.

Primer type	Primers	Size (pb)	Reference
Universal	F: 5'-CAAACTGGGATTAGATACCCCACTAT-3' R: 5'-GAGGGTGACGGGCGGTGTGT-3'	456	Girish et al. (2005)
Bovine	F: 5′-AGCCTGTTCTATAATCGATA-3′ R: 5′-GTTATTGGTTTCATAATAACG-3	221	Aranguren-Méndez et al. (2009)
Pig	F: 5′-AGCCTGTTCTATAATCGATA-3′ R: 5′TTGACATAAATGCTTATCCTATGGC-3′	160	Aranguren-Méndez et al. (2009)
Sheep	F: 5′-AGCCTGTTCTATAATCGATA-3′ R: 5′-GTCTCCTCTCGTGTGGTTGA-3′	364	Aranguren-Méndez et al. (2009)
Goat	F: 5′-AGCCTGTTCTATAATCGATA-3′ R: 5′-CCATGGGTTACACCTTGACC-3′	150	Aranguren-Méndez et al. (2009)

2.3 Restriction fragment length polymorphism

PCR amplicons obtained from the universal primers of the mitochondrial 12S rRNA gene were subjected to restriction enzyme digestion using *AluI* (ThermoFisher) and *ApoI* (Promega), according to the suppliers' instructions. Briefly, enzyme-buffer mix was prepared by mixing 2 µL of restriction enzyme with 8 µL of the respective buffer (Girish et al. 2005). Reaction mix was prepared by mixing 1 µL PCR product with 2.5 µL of enzyme buffer mix. Volume was made up to 20 µL with distilled water. Incubation for *AluI* was carried out at 37 °C for 100 min followed by an inactivation at 65 °C for 15 min. Incubation for *ApoI* was done at 50 °C for 10 min and then, an inactivation at 85 °C for 20 min. Digested product was visualized by electrophoresis in 2% agarose gel along with 100 bp ladder (ThermoFisher).

3 Results and discussion

The mitochondrial 12S rRNA gene fragment was clearly amplified in all samples of the fresh meat by PCR using universal and specific primers. The PCR assay using the universal primers resulted in amplicons of 456 base-pair (bp) in length for all samples of the fresh meat (Figure 1), including mincemeat. The PCR assay using the specific primers (internal control) on the fresh material, resulted in amplicons of 221 bp, 160 bp, 364 bp, and 150 bp for bovine (including mincemeat), porcine, ovine, and goat, respectively (Figure 2). Additionally, PCR using specific primers for goat also amplified 150 bp in ovine meat, revealing their low specificity. Regarding the processed meat, analysis using universal primers generated few amplicons (456 bp); whereas, there were not any amplicons using the specific primers (internal control).

The analysis of the restriction fragments of the amplicons for the fresh meat samples using *AluI* and *ApoI* resulted in different band patterns corresponding to bovine, porcine, and ovine (Table 3), accordingly to Girish et al. (2005), for the six cities in the Amazonas Region (Figure 3 and 4). The patterns of the restriction bands for bovine meat resulted in bands of 359 + 97 bp in length for *AluI* and not any for *ApoI*. The restriction enzymes *AluI* generated 276 + 157 bp for porcine samples, whereas *ApoI* did not generate any restriction bands. The restrictions bands for ovine samples resulted in 246 + 210 bp and 329 + 127 bp for *AluI* and *ApoI*, respectively. The patterns of the restriction bands for the goat meat resulted in the same bands for *AluI* and *ApoI* as ovine samples. These results showed that fresh meat samples correspond to the initial meat species labeling, except for meat samples tagged as goat, in which the restrictions bands agreed with sheep meat. The restriction fragments of the amplicons for processed meat samples using *AluI* generated different electrophoretic patterns (257 + 187 bp, 257 + 156 bp, 365 + 78 bp) (Figure 5). It shows a mix of unidentified species on their composition. There was not any band pattern after digestion using *ApoI*.
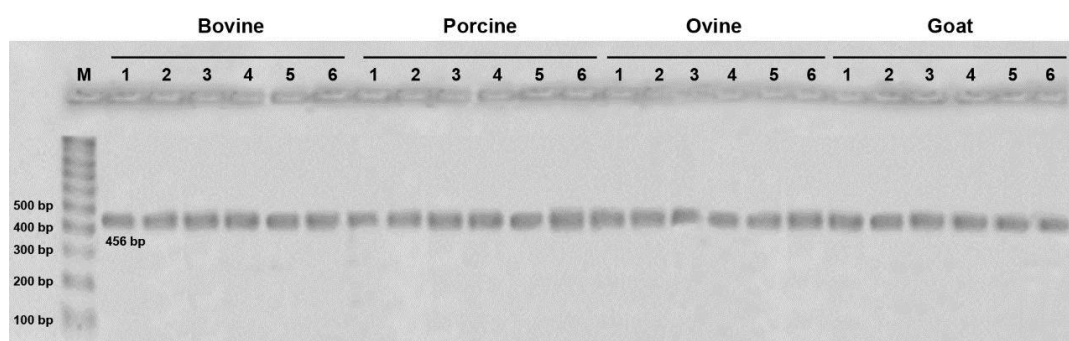

Figure 1. PCR-amplification bands of mitochondrial 12S rRNA gene using universal primers from bovine, porcine, ovine, and goat. (M) 100 bp ladder; (1) Bagua; (2) Bagua Grande; (3) Chachapoyas; (4) Luya; (5) Pedro Ruiz; (6) Rodríguez de Mendoza.
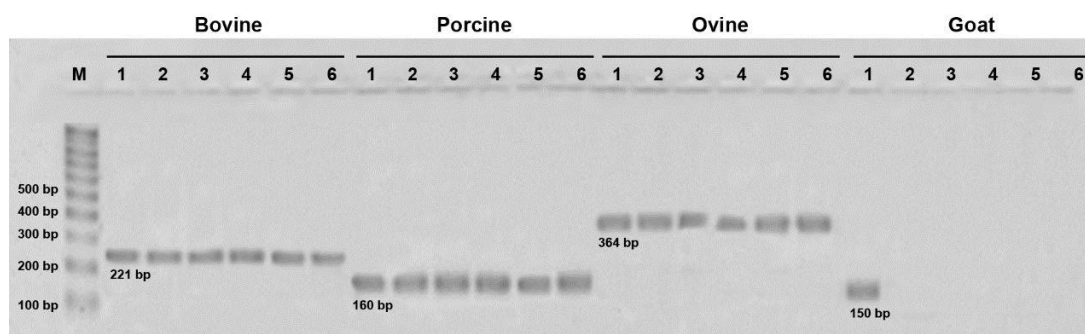

Figure 2. PCR-amplification bands of mitochondrial 12S rRNA gene using specific primers (internal controls) from bovine, porcine, ovine, and goat. (M) 100 bp ladder; (1) Bagua; (2) Bagua Grande; (3) Chachapoyas; (4) Luya; (5) Pedro Ruiz; (6) Rodríguez de Mendoza.
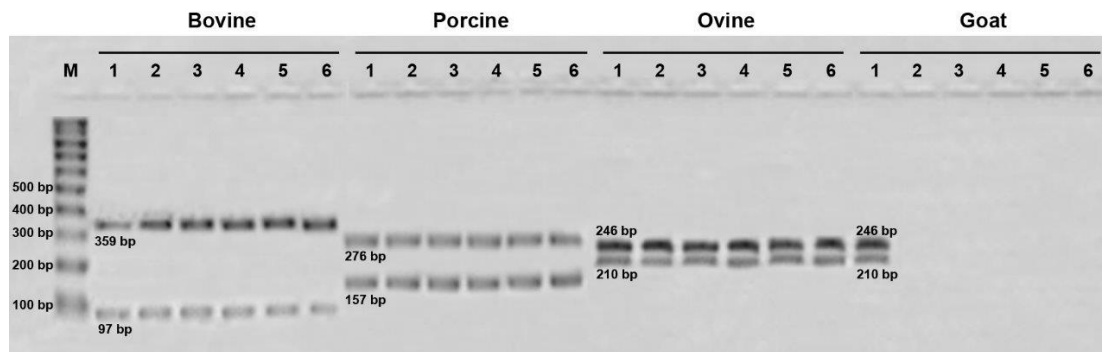
Figure 3. Restriction fragment length polymorphism of mitochondrial 12S rRNA gene of fresh meat products using *AluI* for bovine, porcine, ovine, and goat. (M) 100 bp ladder; (1) Bagua; (2) Bagua Grande; (3) Chachapoyas; (4) Luya; (5) Pedro Ruiz; (6) Rodríguez de Mendoza.

Table 3. Patterns of restriction bands in base pairs (bp) of the restriction enzymes according to Girish et al. (2005).

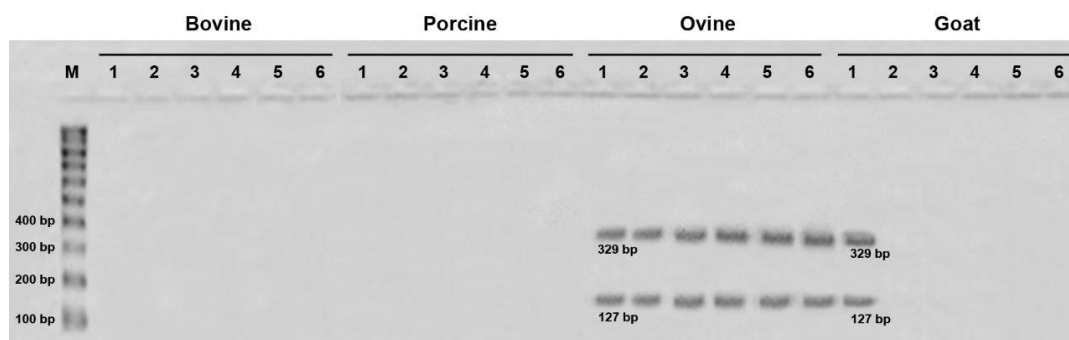| Restriction enzymes	Bovine (Boss taurus)	Porcine (Sus scrofa)	Ovine (Bovis aries)	Goat (Capra aegagrus-hircus)
AluI	359 + 97	276 + 157	246 + 210	246 + 210
ApoI	-	-	329 + 127	-

Figure 4. Restriction fragment length polymorphism of mitochondrial 12S rRNA gene of fresh meat products using *ApoI* for bovine, porcine, ovine, and goat. (M) 100 bp ladder; (1) Bagua; (2) Bagua Grande; (3) Chachapoyas; (4) Luya; (5) Pedro Ruiz; (6) Rodríguez de Mendoza.
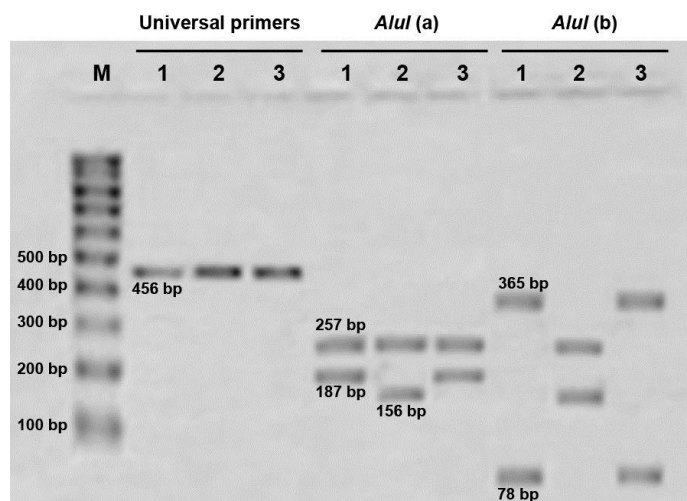

Figure 5. PCR-amplification bands of mitochondrial 12S rRNA gene using universal primers and restriction fragment length polymorphism of mitochondrial 12S rRNA gene of processed meat products using *AluI*. (M) 100 bp ladder; (1) bovine sausage; (2) bovine ham; (3) regional hand-made sausage.

Our amplifications of mitochondrial 12S rRNA gene fragment and posterior digestion by restriction enzymes (*AluI* and *ApoI*) of fresh meat, including mincemeat, demonstrated that there is no evidence of meat substitution, mixing or fraud for bovine, porcine, and ovine species along the six cities. These results are consistent with livestock production in Amazonas, that is the second most important economic activity just after agriculture (e.g. cacao and coffee) (Peru, 2019). This fact assured the meat supply in the region covering the meat demand and thereby, restricting adulteration practices (Sumathi et al., 2015; Sentandreu & Sentandreu, 2014). Conversely, our amplifications of goat samples using specific primers and digestion by restriction enzymes revealed substitution for sheep meat. The limited production of goat meat in the Amazonas Region and its higher prices compared to sheep justified this fraudulent scenario (Arroyo, 2007; Peru, 2019). Additionally, goat is usually confused with "Pelibuey" breed by farmers (Aguilar-Martínez et al., 2017). The Pelibuey is an ovine breed with great adaptability in warm areas, as Bagua city (Aguilar-Martínez et al., 2017).

Regarding the analyses of the processed meat, the absence of amplicons for the mitochondrial 12S rRNA gene using the specific primers (internal controls) in bovine sausage, bovine ham, and regional hand-made sausage, confirmed the lack of bovine, porcine, ovine, or goat species in the composition of these samples. Although processed meat in Amazonas Region is labeled as product of bovine origin, these results confirmed that its meat composition is uncertain. Conversely, digested amplicons of the mitochondrial 12S rRNA gene by the universal primers using the enzyme *AluI* of the processed meats samples generated different electrophoretic patterns (Figure 5) that did not confirm the type of meat used for its manufacture or that there is a mix of species on its composition (Ali et al., 2018). Previous studies pointed out that chilled, frozen and cooked meats did not alter the PCR-RFLP patterns (Sumathi et al., 2015); however, identification of processed meat by PCR-RFLP resulted in subsequent alteration of the restriction fragments due to the DNA degradation by extreme processing conditions as heating, steaming, and radiation during sterilization procedures (Barbosa-Cánovas et al., 2014). Further analyses regarding specificity on the processed meat by including additional molecular markers, restriction enzymes, and specific PCR protocols, should be addressed to determine the meat origin of these samples (Ali et al., 2018).

Our analyses based on samples collected from the Amazonas Region (Bagua, Bagua Grande, Chachapoyas, Luya, Pedro Ruiz, Rodriguez de Mendoza), qualitatively identified and differentiated by PCR-RFLP of mitochondrial 12S rRNA gene three commercial species of fresh meat (bovine, porcine, ovine) and also did not determine the species origin of the processed meat composition.

4 Conclusions

The use of analytical methodologies based on DNA (e.g. PCR-RFLP) to determine the authenticity of different types of meat products after the initial labeling is pivotal to avoid fraudulent practices due to the increased rates of meat consumption. The growth in meat demand in Peru encourages the use of DNA-based approaches to certify the origin of meat products. Monitoring and surveillance with DNA-based analytical methods of meat trade is certainly recommended to determine fraudulent practices as species substitution in markets, especially in regions with bigger populations and higher meat consumption as Lima, Piura, and La Libertad. Moreover, the uncertainty in species origin of processed meat composition based on our PCR-RFLP, suggested its careful consumption since it might be wrongly labeled.

Acknowledgements

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