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Meat species identification by molecular methods: A comprehensive review

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ABSTRACT

The identification of meat species origin is used to ensure food safety, verify labeling compliance, protect consumers against economic fraud, and satisfy socio-religious obligations. Traditional analytical methods targeting morphological traits or proteins fail when applied to highly processed, blended, or thermally degraded meat products. To overcome these limitations, nucleic acid-based molecular techniques have become the gold standard due to the structural stability and ubiquitous presence of DNA across cell types. This review provides a comprehensive synthesis of molecular methods for meat speciation, detailing the history and mechanics of the Polymerase Chain Reaction (PCR), species-specific PCR assays, and Multiplex PCR architectures. The historical development from the early concepts in 1971 to the integration of heat-stable Taq polymerases, followed by an in-depth analysis of targeted genetic markers including the mitochondrial Cytochrome b (cyt b), 12S rRNA, 16S rRNA, D-loop, and Cytochrome C oxidase subunit I (COI) genes.

Keywords: Food fraud, Meat speciation and PCR

1. Introduction

Traceability of meat species and its origin plays a significant role in today's food forensics and quality control. Food adulteration or Fraudulent substitution of meat means mixing of low priced food/meat substituted with high priced food/meats like beef and mutton with lower-cost alternatives like pork, poultry, horse, dog, or cat meat. It affects the people socio-religious cultures like pork consumption is strictly prohibited in Muslim community as well in cow meat consumption in Hindu religion but it's not only violating the people sentiments also effects the financial exploitation.

While historical inspection protocols relied heavily on physical, chemical, anatomical, histological, and immunological and protein based assays such as enzyme-linked immunosorbent assays (ELISA) these procedures exhibit severe vulnerabilities. Processing methods including chopping, mincing, grinding and cooking alter the macroscopic structure of tissues and cause denaturation of antigenic proteins, leaving them undetectable or unquantifiable.

The solutions of the analytical problems solved by DNA-based molecular methods are so popular because a DNA molecule have greater thermal stability and maintains structural integrity than proteins under intensive industrial processing. This review outlines the historical development of these DNA assays, examines specific marker configurations, and contrasts the performance of various simple and multiplex PCR used to authenticate raw meat and processed meat and meat products.

2. Polymerase chain reaction (PCR) in meat speciation

2.1 History and technical evolution of PCR

In 1971, Kleppe and colleagues published a fundamental concept underlying the enzymatic replication of short DNA templates utilizing specific primers in-vitro method was in the Journal of Molecular Biology. Despite its conceptual milestone and innovative nature, this initial demonstration did not gain popularity. The formal invention of the modern Polymerase Chain Reaction (PCR) occurred in 1983 and is universally credited to Kary Mullis.

Early PCR was notoriously tedious because the original DNA polymerases enzymes were degraded at high temperatures. As a result, manually add fresh enzyme to the tube after every single denaturation step. This bottleneck disappeared when researchers isolated a heat-tolerant polymerase in 1976 from *Thermus aquaticus*, a bacterium found in hot springs averaging 50 °C to 80 °C. Because this new enzyme could withstand repeated exposure to boiling temperatures without losing its function, it finally made the automation of ther

mal cyclers possible. To put the power of this breakthrough into perspective, Mullis pointed out that a scientist could use this setup to turn a lone strand of DNA into 100 billion identical copies using just a basic tube, standard reagents, and a controlled heat source.

2.2 Detection of adulteration by standard PCR protocols

The deployment of PCR systems for meat authentication raw, cooked, and highly degraded meat products. According to the Rao *et al.* (1995) [1] verified that genomic DNA extracted from raw muscle could be amplified via PCR for the simple, reliable, and sex-specific identification of domestic animal meats, including buffalo, cattle, sheep, and goat. The scope of meat speciation by molecular techniques across cattle, buffalo, sheep, goat, pig, and chicken meat samples, demonstrated that PCR is a highly informative analytical tool capable of resolving species identity even in heat-treated and putrefied specimens by Thuber (2002) [2].

A critical dimension of molecular tracking involves assessing how matrix degradation affects amplification efficiency. Research by Jain (2007) [3] investigated the variability of the mitochondrial cytochrome b (cyt b) gene in domestic species subjected to severe conditions, including microwave cooking at 100 °C and 120 °C for 30 minutes, alongside natural putrefaction at room temperature for 48 hours. The study revealed that natural decomposition did not inhibit the amplification efficiency of the target cyt b region, demonstrating highly refined detection limits of less than 1 ng of mixed DNA templates. Sindhura *et al.*, (2021) [4] also used targeted mitochondrial cytochrome b (cyt b) gene in mutton and beef samples for detecting meat fraud.

- **12S rRNA Gene:** Rastogi *et al.* (2004) [5] executed species identification and authentication across beef, mutton, chevon, pork, and chicken using mitochondrial 12S rRNA gene sequence analysis paired with conformation sensitive gel electrophoresis, achieving an detection of adulteration up to 10% in heat-treated products. Similarly, Girish *et al.* (2005) [6] applied PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) to a 456-bp amplified fragment of the 12S rRNA gene to identify across meats like beef, buffalo, mutton, and chevon. While their PCR-RFLP assay provided consistent results for individual fresh and processed samples, it faced limitations when processing complex multi-species meat samples. Mahajan *et al.* (2011) [7] also used 12S rRNA gene via PCR-RFLP to differentiate beef, buffalo meat, chevon, and mutton. Their system successfully resolved binary species mixtures at ratios of 50:50 and 75:25 (except for chevon-beef blends), though it struggled to detect contaminants at a 90:10 ratio, unless distinguishing between cattle and buffalo.

- **16S rDNA and Nuclear Markers:** Rastogi *et al.* (2007) [8] conducted a comparative performance evaluation between mitochondrial markers (16S rDNA and NADH dehydrogenase subunit 4 (ND4) and nuclear markers (Actin gene) across domestic meat species. Finally concluded that mitochondrial markers plays a key role on species validation due to their higher copy numbers per cell, which helps mitigate the effects of processing-induced degradation.

- **D-loop Region:** Mane *et al.* (2009) [9] engineered a highly specific primer pair targeting the mitochondrial D-loop gene to detect chicken contamination. The assay successfully amplified a 442-bp fragment from fresh, processed, and autoclaved meat products without any loss of efficiency caused by thermal processing, achieving a detection sensitivity below 1% in admixed configurations.

- **Real-Time PCR Diagnostics:** Kumari (2007) [10] designed a Real-Time PCR platform to differentiate domestic meats, focusing particularly on cattle and buffalo. By pairing a duplex PCR with a cattle-specific Real-Time PCR assay, the system achieved a highly precise detection limit, identifying cattle meat mixed into buffalo meat down to a 1:1000 fraction. A specialized duplex PCR molecular study was demonstrated by Sindhura *et al.* (2021) [4] to detect food adulteration of raw meat mutton with beef.

- **Single-Nucleotide Primer Extension (Snapshot Assay):** Gupta *et al.* (2011) [11] leveraged single-nucleotide primer extension within the mitochondrial cyt b region to authenticate cattle and buffalo meat.

This Snapshot assay successfully identified 1% adulteration levels in mixed samples and maintained high performance across fresh, cooked, and putrefied specimens, proving that the technique remains highly effective even when analyzing severely degraded DNA.

3. Species-specific PCR

Species-specific PCR relies on oligonucleotides designed to hybridize with unique sequence motifs inside a targeted gene, eliminating the need for subsequent enzymatic digestion or sequencing steps. Frezza *et al.* (2008) [12] demonstrated that species specific PCR approach is highly specific, rapid, and cost-effective for routine food authentic labs.

Lahiff *et al.* (2001) [13] conducted species-specific assays to detect ovine, porcine, and poultry fractions within industrial meat and bone meal (MBM). They validated their PCR results using restriction endonucleases, using HphI for cattle, MnlI for pig, HindIII for chicken, and SspI for ovine targets.

To detect multi-species fraud, Ilhak *et al.* (2007) [14] analyzed complex minor meat mixtures by adding varying concentrations (5%, 2.5%, 1%, 0.5%, and 0.1%) of pork, horse, cat, or dog meat into base matrices of beef, sheep, and goat. Their species-specific PCR assay successfully identified the target components by generating unique mitochondrial DNA amplicons: Horse (439 bp), Dog (322 bp), Cat (274 bp), Bovine (271 bp), Sheep (225 bp), Porcine (212 bp), and Goat (157 bp).

Thermal resilience was further demonstrated by Karabasanavar *et al.* (2011) [15], who designed specific primers targeting the mitochondrial D-loop gene to identify raw and heat-treated sheep meat. The assay generated clear 329-bp and 404-bp fragments, showing no cross-amplification when tested against 25 different reference species. The system maintained its diagnostic accuracy across raw, cooked (60 °C, 80 °C, and 100 °C for 30 minutes), and autoclaved samples (121 °C for 30 minutes), achieving a Limit of Detection (LOD) of 1 picogram of pure ovine DNA.

Additionally, Kesmen *et al.* (2012) [16] successfully applied species-specific PCR assays to cooked commercial meat sausages, demonstrating that complex spice mixtures, chemical ingredients, and industrial processing do not affect the amplification of target muscular DNA.

4. Multiplex PCR architectures

Multiplex PCR is highly benefited to large scale sample processing because it's rapid, saves time and reagents requirements also minimal. It improves upon traditional single-target PCR by introducing multiple primers into a single reaction tube, enabling the concurrent amplification and detection of several species. As highlighted by Tauma *et al.* (2014) [17], the capability to screen for multiple target sequences in a single reaction has led to the widespread adoption of multiplex PCR in both industrial testing and academic research.

4.1 Development and screening capability

An early foundation for multiplexing was established by Matsunaga *et al.* (1999) [18], who developed a multiplex PCR assay to simultaneously screen six meat types: beef, horse, sheep, goat, pig, and chicken. The system successfully detected adulteration even after samples were heated at 100 °C or 120 °C for 30 minutes, maintaining a detection sensitivity limit of 0.25 ng of total template DNA.

Dalmaso *et al.* (2004) [19] focused on rendering plant verification by designing a multiplex assay to monitor livestock feed components (ruminant, poultry, fish, and pork) using primers distributed across the mitochondrial 12S rRNA, tRNA-Val, and 16S rRNA genes. Similarly, Ghovvati *et al.* (2009) [20] used conserved regions of the 12S and 16S rRNA genes to evaluate commercial food products, including ground meat, sausages, and cold cuts. Their screening revealed that while none of the samples contained undeclared porcine materials, 40% of the sausages and 30% of the cold cuts were contaminated with undeclared poultry fractions.

To protect premium game meat supply chains, Zha *et al.* (2010) [21] developed a one-step multiplex PCR assay to detect common adulterants (bovine, ovine, porcine, and poultry) in deer products by targeting the mitochondrial tRNA-Val and 16S rRNA genes.

4.2 Diagnostic amplicon matrices and socio-religious contexts

A key advantage of multiplex PCR is its ability to separate distinct species based on the size of their amplified DNA fragments (amplicons) when analyzed via gel electrophoresis. Ashwani *et al.* (2009) developed a cyt b multiplex assay to distinguish between cooked meat of sheep (331 bp), goat (157 bp), cattle (274 bp), pig (398 bp), and chicken (227 bp). They emphasized that clear differentiation between cattle and pig components is particularly important for satisfying socio-religious dietary requirements.

Similarly, Makwana *et al.* (2016) [22] used a cyt b gene approach with a shared forward primer paired with species-specific reverse primers to generate distinct diagnostic fragments for goat (157 bp), chicken (227 bp), buffalo (274 bp), and sheep (331 bp). For regional culinary authentication, Bhat *et al.* (2016) [23] developed a cyt b multiplex assay to detect the adulteration of traditional Kashmiri Wazwan cooked mutton Rista with beef or buffalo meat, yielding distinct fragments for buffalo (124 bp), cattle (472 bp), and sheep (585 bp).

5. Deep-dive analysis of extraction frameworks and matrix interferences

To truly understand why molecular diagnostics achieve such precision, we must look closely at the initial step: extracting the template DNA from the matrix. The physical state of a commercial meat sample profoundly shapes its physical and chemical properties. In a laboratory setting, when handling composite foods like sausages, ready-to-eat [REF] foods, or animal feedstuffs, the target material is rarely a pristine piece of muscle tissue. Instead, it is a complex mixture of lipids, proteins, texturizers, binding agents, and chemical additives.

5.1 Mechanical disruption and lysis dynamics

The extraction process begins with thorough physical disruption. Liquid nitrogen freezes the tissue rapidly, making it brittle and locking the biochemical profile in place. This prevents endogenous nucleases from breaking down the genetic material before extraction buffers can deactivate them. Once ground, the sample enters a lysis buffer, typically containing an ionic surfactant like Sodium Dodecyl Sulfate (SDS) or a non-ionic surfactant like Cetyl-tri-methyl ammonium Bromide (CTAB), paired with a robust proteolytic enzyme, most commonly Proteinase K was used. SDS breaks down cell membranes by disrupting the lipid bilayer and unfolding cellular proteins, while CTAB is especially useful for high-fat or plant-adulterated matrices because it forms complexes with polysaccharides and complex molecules, separating them from the nucleic acids.

5.2 Phenol-chloroform extraction vs. silica-based spin columns

The most common recovery benchmark was phenol-chloroform-isoamyl alcohol mixture. In this method, it separates the samples in different phases based on the density and polarity. Proteins denature during centrifugation and are found in the pellet at the interface of the organic and aqueous phases, lipids distribute into the lower organic phase, and very pure nucleic acids remain in the upper aqueous layer. Although this classical protocol yields large amounts of high molecular weight DNA it bears major operational disadvantages. The chemicals are very toxic, any residual phenol or chloroform will effectively inhibit the polymerase enzyme during PCR. To counter these problems, modern routine testing laboratories have switched to silica-gel

membrane spin columns or automated magnetic bead system. A silica column depends on chaotropic salts, like guanidine hydrochloride, to dehydrate the DNA backbone, forcing it to bind selectively to the silica matrix while proteins and sugars are washed away. Once clean, a low-ionic-strength buffer releases the purified DNA, providing a highly reliable template for downstream applications.

5.3 Chemical and physical inhibitors in processed food matrices

Potent inhibitors can be introduced during industrial processing that interfere with enzymatic assays. For example, cured meats frequently contain significant quantities of sodium chloride, sodium nitrite and several polyphosphates. Also, polyphenols, humic acids and complex polysaccharides in seasoning agents such as black pepper, garlic powder and complex spice mixes can survive standard extraction protocols. These molecules either bind directly to the Taq polymerase enzyme or compete for the magnesium ions, which are essential co-factors for the amplification reaction. This can lead to false negatives especially in the case where the search is for low levels of an adulterant. More sophisticated protocols have added polyvinylpyrrolidone (PVPP) to the mix to bind and remove the polyphenols, or used extended alcohol washing steps to ensure that the final DNA template is free of chemical residues.

6. Advanced innovations in molecular testing paradigms

The technological advancements were changing the shape of forensics by past conventional methods to present qualitative Multiplex procedures. These developments move away from simple qualitative screening, pushing toward absolute quantification and decentralized field testing.

6.1 Digital droplet PCR (ddPCR) for absolute quantitation

A standard real-time PCR gives on standard curves generated from reference samples to determine concentration. Digital Droplet PCR (ddPCR) avoids this requirement by splitting a single 20-microliter reaction into roughly 20,000 separate, nanoliter-sized water-in-oil droplets. Each droplet acts as an independent reaction chamber, containing either zero, one or multiple copies of the target DNA fragment. Applying Poisson statistics to the ratio of positive (fluorescent) to negative (dark) droplets gives the absolute concentration of target DNA without requiring external reference standards. This level of accuracy is more important to detect the meat fraud between intentional and unintentional financial fraud, cross-contamination that can occur in shared industrial processing line.

6.2 Isothermal amplification platforms: LAMP and RPA

A traditional method of PCR-based testing is the need for sophisticated, energy-intensive thermal cyclers to handle rapid temperature shifts. This requirement restricts testing to centralized laboratories. Isothermal amplification methods fix the DNA at a single, constant temperature. Loop-Mediated Isothermal Amplification (LAMP) uses four to six specially designed primers paired with DNA polymerase, typically derived from *Bacillus stearothermophilus*. The reaction operates between 60 °C and 65 °C, generating complex loop structures that produce massive amounts of DNA in under an hour. This high production rate often creates a dense magnesium pyrophosphate precipitate, which can be seen by the naked eye or measured with simple colorimetric dyes. Similarly, Recombinase Polymerase Amplification (RPA) utilizes specialized bacterial proteins to insert primers directly into double-stranded DNA at room temperature (37 °C to 42 °C). Because these tests require only a basic water bath and it's easy for rapid, on-site species verification directly at inspection points.

Target Genes / Markers	Species Covered	Fragment Sizes (bp)	Sensitivity / Detection Limits	Reference (s)
16S rRNA	Poultry, Fish, Pork, Ruminant	183, 224, 290, 374	Identified unlabeled poultry in commercial red meat products.	Sakalar & Abasiyanik (2011)
cyt b and ND5/6	Chicken, Pig, Cow, Crocodile	350, 570, 750, 1000	Down to 5 ng of total template DNA from meat or blood.	Unajak <i>et al.</i> (2011)
Mitochondrial cyt b	Buffalo, Goat, Cattle, Sheep	Species-specific array	Capable of tracing less than 10% of contaminant species in mixtures.	Zarringhabaie <i>et al.</i> (2011)
Species-specific loci	Mutton, Cattle	331 (Mutton), 247 (Cattle)	Successful traceability of non-descript sheep meat.	Babu <i>et al.</i> (2014)
Direct PCR Matrix	Pork, Lamb/Mutton, Chicken, Ostrich, Horse, Beef	100, 119, 133, 155, 253, 311	High performance across heavily degraded and processed matrices.	Kitipit <i>et al.</i> (2014)
Islamic Prohibited Loci	Five prohibited meat species	Multiplexed profiling array	0.01–0.02 ng pure DNA; 1% contaminant in meatball formulations.	Ali <i>et al.</i> (2015)
t.glu + cyt b, 12S rRNA, ATPase 8, 18S rRNA	Ovine, Caprine, Fish, Bovine, Eukaryotic control	119, 142, 224, 271, 99 (Control)	Rapid validation of multi-species components in animal feedstuffs.	Safdar & Junejo (2015)
Pentaplex direct assay	Pork, Chicken, Beef, Duck, Eukaryotic control	83, 133, 166, 204	Zero cross-reactivity when evaluated against 16 reference species.	Kim & Kim (2017)
Cytochrome C Oxidase I (COX-1)	Pig, Camel, Sheep, Donkey, Goat, Cow, Chicken	Single-tube separate bands	Fully distinguishes seven species origins simultaneously.	Izadpanah <i>et al.</i> (2018)
Cytochrome Oxidase I (COI)	Buffalo, Cattle, Pork, Duck	313, 255, 294, 177	1 pg pure DNA; 0.1% (w/w) adulterated meat under mixed matrices.	Wang <i>et al.</i> (2018)

6.3 Next-generation sequencing (NGS) and metabarcoding solutions

When working with highly complex, multi-component commercial products like pet foods, mixed sausages, or rendering plant bone meals, targeted PCR assays are limited by the need to select specific target species beforehand. Next-Generation Sequencing (NGS) combined with *meta*-barcoding removes this limitation. Instead of relying on species-specific primers, metabarcoding uses universal primers to amplify a highly conserved gene region, such as the 16S rRNA or Cytochrome Oxidase I (COI) gene, across all organisms present in the sample. High-throughput sequencers then read millions of these amplified fragments simultaneously. Comparing these sequences against comprehensive bioinformatic reference databases like GenBank or BOLD reveals the complete biological makeup of the sample. This unguided approach can identify unexpected adulterants, exotic bushmeats, or undeclared plant fillers that standard targeted assays would completely miss.

7. Socio-religious impacts, global supply chain auditing, and regulatory standards

The technical details of meat speciation are directly tied to deep social, ethical, and economic concerns. Food choices are intimately connected to culture, personal health, and faith, making accurate labeling a fundamental consumer right protected by complex international laws.

7.1 Halal and kosher food integrity frameworks

For billions of consumers globally, identifying specific meat species is a matter of strict religious compliance rather than simple economic value. Islamic dietary laws (Halal) strictly prohibited consuming pork meat derivatives, while Jewish dietary laws (Kosher) enforce detailed rules regarding permissible species and slaughter methods. In these frameworks, even minor cross-contamination from pork or unpermitted species can render an entire manufacturing facility non-compliant. Consequently, molecular testing methods must achieve exceptional sensitivity. The multiplex systems detailed by [Ali et al. \(2015\)](#) [24] and [Ashwani et al. \(2009\)](#) [25] provide the high reliability needed to verify these lines, ensuring that trace residues of prohibited species are detected even within complex formulations.

7.2 Global supply chain auditing and economic fraud mitigation

Modern food supply chains are highly complex and globalized. A single processed meat product might contain ingredients sourced from multiple countries, passing through numerous intermediaries, cold

storage facilities, and processing plants. This complexity creates opportunities for fraudulent substitution of expensive meats with cheaper alternatives, or to blend low-grade animal tissues into premium products. By introducing routine molecular testing at critical control points throughout the supply chain, companies and regulatory bodies can establish transparent, verifiable traceability. This active oversight protects honest producers from unfair competition and helps rebuild consumer trust in commercial food labels.

7.3 International regulatory guidelines and validation protocols

To ensure testing consistency across international borders, organizations like the Association of Official Analytical Chemists (AOAC), the European Committee for Standardization (CEN), and Codex Alimentarius publish strict validation protocols. These guidelines require assays to demonstrate clear precision, specificity, and robust performance across different laboratory environments before they are approved for official regulatory enforcement. These standardized criteria ensure that a molecular test performed at an export shipping port will yield identical results when re-tested by customs authorities at an import destination, creating a reliable, science-based foundation for international food trade.

8. Technical summary of molecular targets

To provide a clear overview of the genetic markers discussed throughout this literature review, the following matrix compares the main mitochondrial and nuclear targets used in contemporary food forensics.

9. Conclusion

DNA-based molecular methods have fundamentally transformed food authentication and forensics. By targeting multi-copy mitochondrial loci such as *cyt b*, 12S rRNA, 16S rRNA, *D*-loop, and COI, these techniques provide the high sensitivity and specificity required to detect minor contaminants in complex food matrices. Their structural resilience ensures reliable performance even when analyzing samples subjected to intensive thermal processing, micro-waving, autoclaving, or natural putrefaction.

As technologies continue to advance from single species-specific PCR setups to highly multiplexed direct-amplification systems, digital droplet PCR platforms, and unbiased next-generation sequencing approaches molecular diagnostics remain essential tools for enforcing food safety standards, preventing economic fraud, and ensuring compliance with socio-religious dietary laws across the global supply chain.

Genetic Marker	DNA Source	Copy Number / Cell	Evolutionary Rate	Primary Analytical Application
Cytochrome b (<i>cyt b</i>)	Mitochondrial	High (Hundreds to Thousands)	Rapid Mutation Rate	Universal species identification; robust under extreme thermal degradation.
12S rRNA Gene	Mitochondrial	High (Hundreds to Thousands)	Moderate / Conserved	Broad taxonomic discrimination; excellent for PCR-RFLP profiles.
16S rRNA Gene	Mitochondrial	High (Hundreds to Thousands)	Moderate / Conserved	Differentiating deep evolutionary lineages; key target for animal feedstuffs.
<i>D</i> -loop Region	Mitochondrial	High (Hundreds to Thousands)	Very Rapid (Hypervariable)	Distinguishing closely related species or regional breeds; high sensitivity.
Cytochrome Oxidase I (COI)	Mitochondrial	High (Hundreds to Thousands)	Rapid Mutation Rate	Standard universal DNA barcoding database alignment across all animal taxa.
Actin Gene	Nuclear	Low (Two copies per diploid cell)	Slow Mutation Rate	Internal amplification control; quantitative assessment of tissue mass components.

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