JOURNAL OF FOOD QUALITY & HAZARDS CONTROL
SHAHID SADOUGHI UNIVERSITY OF MEDICAL SCIENCES & HEALTH SERVICES

Journal of Food Quality and Hazards Control 8 (2021) 152-161

Tracing the Pig and Cattle Origin in Processed Food and Feed Products Targeting Mitochondrial 12S rRNA Gene

D.C. Roy [1*], S. Akhter [1], A.K. Sarker [1], M.M.K. Hossain [2], C. Lyzu [1], L.C. Mohanta [1], D. Islam [1], M.A.A. Khan [1]

1. Biomedical and Toxicological Research Institute, Bangladesh Council of Scientific and Industrial Research, Dhanmondi, Dhaka-1205, Bangladesh
2. Animal Biotechnology Division, National Institute of Biotechnology, Ganakbari, Ashulia, Savar, Dhaka-1349, Bangladesh

HIGHLIGHTS

- Amplification requires the presence of mtDNA in 0.001 ng of pig and 0.01 ng of cattle total DNA.
- The most intense expected DNA bands of pig and cattle were produced at 50 °C.
- Specificity of the primer pairs ensures the pig/cattle tracing in food and feed stuffs.

Article type
Original article

Keywords
Fraud
Food Analysis
DNA, Mitochondrial
DNA Primers
Swine
Cattle

Article history
Received: 29 Jul 2021
Revised: 22 Oct 2021
Accepted: 3 Nov 2021

Acronyms and abbreviations
MBM=Meat and Bone Meal
mtDNA=Mitochondrial DNA
PCR=Polymerase Chain Reaction

ABSTRACT

Background: Species identification in commercially processed food and feed products is one of the important issues. This study was conducted to develop a genetic method for the detection of pig and cattle species in processed food and feed products using newly designed species-specific primers targeting mitochondrial 12S rRNA gene fragments.

Methods: Two sets of specific primers were designed based on the 12S rRNA gene sequences of pig and cattle species from GenBank. The primers were validated by using the DNA extracted from nine different chordates, including pig, cattle, chicken, bata fish, bat, toad, African parrot, rat, and human origin. Annealing temperature ranging from 46-54°C for 30 seconds and template DNA 1:10 serial dilutions ranging from 10 to 0.00001 ng/µl were employed for primer annealing and sensitivity analysis. Samples were analyzed using optimized Polymerase Chain Reaction (PCR) conditions.

Results: The most intense expected DNA bands of pig and cattle were produced at 50°C. Under that optimized annealing temperature pig and cattle-specific primers did not anneal with the DNA of other chordates. Total extracted DNA 0.001 ng and 0.01 ng of pig and cattle respectively containing the mitochondrial DNA (mtDNA) was successfully detected.

Conclusion: These findings indicate that the newly designed primer pairs can be used to detect pig and cattle derivatives in various processed food and feed products.

© 2021, Shahid Sadoughi University of Medical Sciences. This is an open access article under the Creative Commons Attribution 4.0 International License.

Introduction

Adulteration or preparation of food and feed products by combining meats and fats of various cheaper origins along with ambiguous and improper labeling is a common practice in most countries. These types of false labeling, adulteration with cheaper species or against religious specifications, are of major concern for many consumers (Nehal et al., 2021). For instance, in Bangladesh feed products like meat and bone meal (MBM) are

* Corresponding author (D.C. Roy)
✉ E-mail: roydc1987@gmail.com
ORCID ID: https://orcid.org/0000-0003-0299-5710

To cite: Roy D.C., Akhter S., Sarker A.K., Hossain M.M.K., Lyzu C., Mohanta L.C., Islam D., Khan M.A.A. (2021). Tracing the pig and cattle origin in processed food and feed products targeting mitochondrial 12S rRNA gene. *Journal of Food Quality and Hazards Control*. 8: 152-161.

DOI: 10.18502/jfqhc.8.4.8256 Journal website: http://jfqhc.ssu.ac.ir

[Downloaded from jfqhc.ssu.ac.ir on 2025-05-20]
[DOI: 10.18502/jfqhc.8.4.8256]

strictly prohibited by the Import Policy Order 2015-2018 (The Import Policy Order of Bangladesh 2015-2018), as MBM is mostly prepared from pig and cattle leftovers. The pig is prohibited for the Muslim community (Che Man et al., 2012) and on the other hand, cattle origins have the risk of bovine spongiform encephalopathy and transmissible spongiform encephalopathy (Costassa et al., 2016). Again human consumable food products may also be replaced partially or fully by the undesired species or lower valued species by fraud manufacturers for economic gain (Johnson, 2014). Interspecies food and feed products adulteration or preparation by mixing meats and fats of different origin can be a threat to safety or negatively affect the nutritional performance (Tibola et al., 2018). Thus, confirming the species authenticity of food and feed products is a national issue to comply with the regulations and to sustain the products in global competition (Di Pinto et al., 2015). In addition, determining the origin of the meat species is a valuable method for evaluating the risk associated with the introduction of animal content that may be harmful to human or animal health (Hutasoit et al., 2021; Rojas et al., 2011).

Several methodologies have been suggested so far for the detection of organisms in complex mixtures, primarily based on protein and DNA analysis. DNA-based Polymerase Chain Reaction (PCR) methods of species identification and authentication have gained popularity over protein-based approaches. Because DNA is more stable in both fresh raw and processed food and feed products than proteins (Amaral et al., 2015). Moreover, most of the methods for identifying the species origin in food and feed products are based on PCR due to their high specificity and sensitivity and rapid processing time (Fajardo et al., 2010; Izadpanah et al., 2018; Kumar et al., 2015).

In evolutionary research and species identification, mitochondrial DNA (mtDNA) regions are frequently employed as molecular markers because of their higher specificity (Amorim et al., 2019; Barakat et al., 2014; Fang and Zhang, 2016; Ricardo et al., 2020). This approach is more sensitive in identifying closely related species than nuclear DNA. Because each cell has only one set of genomic DNA in the nucleus but 104 copies of mtDNA with a high mutation rate due to low polymerase correction systems (Amorim et al., 2019; Khanzadeh et al., 2020; Zhang et al., 2021). Using the PCR methods, it is possible to detect the species-specific mtDNA even in pg present in both processed and unprocessed samples. Species-specific designed primers from the different genes like D-loop, 12S rRNA, 16S rRNA, cytochrome oxidase I of mtDNA are usually for species identification (Cho et al., 2014). Besides having the variable regions, these genes also contain the conserved regions. Primers designed from the variable regions are usually specific to the species and only the traditional PCR rather than sequencing or Restriction Fragment Length Polymorphism (RFLP) analysis are used for species identification (Cho et al., 2014; Izadpanah et al., 2018). However, deep Primer-BLAST filtering of several of the frequently used pig and cattle-specific primers reported in the research of Cahyadi et al. (2018) and Wang et al. (2019) demonstrates that they are false specific. In this perspective, pig and cattle species-specific primer design from the hyper variable regions along with the validation of PCR conditions for respective origin identification in food and feed products may be an effective approach.

Thus, the main objective of this work is to design the pig and cattle species-specific primers targeting mitochondrial 12S rRNA gene fragment and to detect pig and cattle origin in food and feed products by a simple band identification of PCR products.

Materials and methods

Primer design

The species-specific primer pairs were designed and selected through online based primer-BLAST of NCBI (link: https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). A number of primer pairs targeting the 12S rRNA gene were returned, considering at least 2 total mismatches to unintended targets, including at least 2 mismatches within the last 5 bps at the 3' end and ignoring targets with 6 or more mismatches. Checking them through online based primer-BLAST of NCBI with the different species of chordates only the best species-specific primer pairs were selected. Then the primer sequences having comparatively lower self complementarity and self 3' complementarity value were checked by online based Multiple Primer Analyzer tool of Thermo Fisher Scientific platform to avoid primer dimer formation. Finally, the selected primer pairs shown in Table 1 were checked with the other species under the same genus/family. The frequently occurring degenerate bases were compressed using nucleic acid notation formalized by the International Union of Pure and Applied Chemistry (IUPAC). Synthesized newly designed primers were purchased from Integrated DNA Technologies Pte. Ltd., Singapore.

Samples

The fresh raw meat sample of pig (*Sus scrofa domesticus*) from the Christian missionary in Dhaka city; and cattle (*Bos taurus*), chicken (*Gallus gallus domesticus*), bata fish (*Labeo bata*), sheep (*Ovis aries*) meat purchased from local wet markets in Dhaka city, Bangladesh. Bat (*Pipistrellus coromandra*) and toad (*Duttaphrynus melanostictus*) meat were collected from

[Downloaded from jfqhc.ssu.ac.ir on 2025-05-20]

[DOI: 10.18502/jfqhc.8.4.8256]

nature. Fresh feathers of an African parrot (*Psittacus erithacus*) were provided by a bird seller. Laboratory bred rat (*Rattus norvegicus*) meat and human blood respectively provided by Animal Resource Division and Biochemistry and Molecular Biology Research Division, BTRI, BCSIR, Dhaka. Imported brand food and feed samples were supplied by the food and feed importers through the Custom House. The samples were stored at -20 °C until used for DNA extraction.

Total DNA extraction

Respective total DNA from approximately 25 mg of meat, blood, feathers, food, and feed samples were extracted using PureLink™ Genomic DNA Mini Kit (USA) as per the instructions given by the manufacturer with some modifications. The modifications include digestion of the crushed bone samples for more than 4 h and addition of 200 µl 100% isopropanol to the particulate materials free digested lysate rather than ethanol. The DNA concentration was measured using fluorescence-based dyes by Qubit 3.0 fluorometer (applied biosystems, Malaysia). The extracted DNA solution was stored in −20 °C until further use.

Thermal cycling conditions optimization

The reactions were performed in a thermal cycler (Applied Biosystems™ 2,720 Thermal Cycler, Singapore). The cycling conditions *i.e.* cycling times and temperatures were determined based on the based on the size of the template and the GC content of the DNA, DNA polymerase and melting temperatures of primers as follows: initial denaturation step at 95 °C for 5 min, 40 cycles of amplification (30 s at 95 °C, 30 s at various annealing temperature range from 46 °C to 54 °C, extension at 72 °C for 35 s), followed by final extension at 72 °C for 7 min.

The annealing temperature optimization through PCR amplifications of pig and cattle-specific fragments in 12S rRNA gene was accomplished individually in a total volume of 10 µl. Each 10 µl reaction mix contains 5 µl GoTaq® G2 PCR Master Mix (2×), 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM), 1 µl DNA Template (100-150 ng/µl), and 3 µl nuclease free water. In order to confirm the purity of the PCR reaction mixture from contaminating DNA, no template control containing no template DNA but an equal amount of nuclease free water was used.

Amplified products were analyzed by electrophoresis at 90 volts in 1×Tris-Acetate-EDTA (TAE) buffer in 2% agarose gel. Agarose gels post staining with ethidium bromide were visualized using Fluorescence UV-Vis Imaging system (BIO-PRINT TX4, France).

Primer sensitivity testing

Under the optimized PCR conditions, the detection limit of pig and cattle-specific primers was determined by amplification of a 1:10 serial dilution of pig/cattle DNA. In each PCR reaction volume 1 µl of 10, 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001 ng/µl serially diluted pig or cattle DNA templates were added independently to make each final volume to 10 µl. Electrophoresis analysis of amplified PCR products was examined as done under section of thermal cycling conditions optimization.

Primer specificity testing

Specificity was tested through optimized PCR of spiked and non-spiked pig or cattle DNA in the DNA admixture of nine chordate species, such as bat, bata fish, chicken, human, parrot, rat, sheep, toad, and pig/cattle using the same proportion of PCR reaction mixture. Amount of DNA for spiking was determined from the sensitivity testing. Each 1 µl of 200 ng/µl DNA admixtures of nine chordates was spiked with 1 µl of 10, 1, 0.1, 0.01, 0.001, and 0.0001 ng/µl DNA of pig or cattle. Non-spiked DNA admixtures were amended by equivalent amounts of nuclease free water instead of pig or cattle DNA. Amplified PCR products were analyzed through electrophoresis.

Primer pairs applying in industrially processed sample testing

Extracted DNA from industrially processed samples such as MBM, crushed bones, fish meal, poultry meal, vegetable fat, and milk cream supplied by importers through Custom House was employed for authentication. As the industrially processed samples undergo a number of treatments, inhibitory materials may be added to any stage of processing. Thus, one part of each sample was independently spiked by 1 µl (10 ng/µl) pig/cattle DNA. Note that, those samples were previously analyzed using the primers stated in the study of Spychaj et al. (2016).

Results

Virtual properties of newly designed primer pairs

Using NCBI's web based primer-BLAST, the sequence homology search targeting 12S rRNA gene, pig and cattle-specific primers revealed considerable homology to eight different pig and cattle species (Table 1). It further verified that the primer pairs are exclusive to pigs and cattle, with no sequence homology with other species. The size of pig-specific fragments was projected to be 520-523 bps, whereas cattle-specific fragments were expected to be 179 bps.

[Downloaded from jfqhc.ssu.ac.ir on 2025-05-20]

[DOI: 10.18502/jfqhc.8.4.8256]

Effects of different annealing temperature on amplification of 12S rRNA gene

Pig and cattle-specific fragments of 522 and 179 bps respectively amplified from 12S rRNA gene at annealing temperatures ranging from 46 °C to 54 °C, as shown in Figures 1 and 2. The most intense band was produced in both cases at 50 °C annealing temperature.

Primer sensitivity to target template DNA concentration

The DNA bands were found to fade gradually when the concentration of DNA template was reduced. The high sensitivity conferred by the pig and cattle-specific assay was proved by successful amplification of as low as 0.001 ng pig DNA (Figure 3) and 0.01 ng cattle DNA (Figure 4). Below these concentrations no bright DNA bands but blurred band-like appearances were observed.

Primer specificity over other species

The assay utilizing the pig or cattle-specific primers were cross-tested with the admixture of several animal species (bat, bata fish, chicken, human, parrot, rat, sheep, toad, and pig/cattle). As expected, the 522 and 179 bps band, amplicons specific to the pig and cattle respectively, were not produced in all other animal species tested (Figures 5 and 6). Furthermore, DNA admixture of other species when spiked with different concentrations of pig or cattle DNA, up to 0.01 ng DNA of both pig and cattle produced only the expected bands. There were no other impurities found, such as product degradation, primer-dimer formation, and presence of contamination.

Optimized PCR conditions in industrially processed sample analysis

No specific target DNA band was found during analysis of MBM, crushed bones, fish meal, poultry meal, vegetable fat, and milk cream samples (Figure 7). On the other hand, except crushed bones, fish meal, poultry meal, and vegetable fat, DNA of the other 3 samples produced visible DNA bands (Figure 8). All the samples spiked with pig or cattle DNA were amplified precisely.

Table 1: Pig and cattle specific primer sequences

Primer Name	Sequence (5´➤3´)	Length (b)	T_m (°C)	Product Size (bps)	Detectable Species
SS12S520-3F	TCTAATAAT**Y**CTGACACACG	20	48.0	520-523	**Pig species** (*Sus scrofa, Sus cristatus, Sus taivanus, Sus celebensis, Sus verrucosus, Sus barbatus, Sus cebifrons, Babyrousa babyrussa*)
SS12S520-3R	GCATGGTTGTGTAATT**R**AATAT	22	48.2		
BT12S179F	CTCTACAC**Y**AAGAGAA**Y**CAAG	21	49.9	179	**Cattle species** (*Bos taurus, Bos primigenius, Bos indicus, Bos javanicus, Bos frontalis, Bos gaurus, Bison priscus, Bison bison*)
BT12S179R	GTAGCTAGTGC**R**TTTAAATA**R**G	22	49.0		

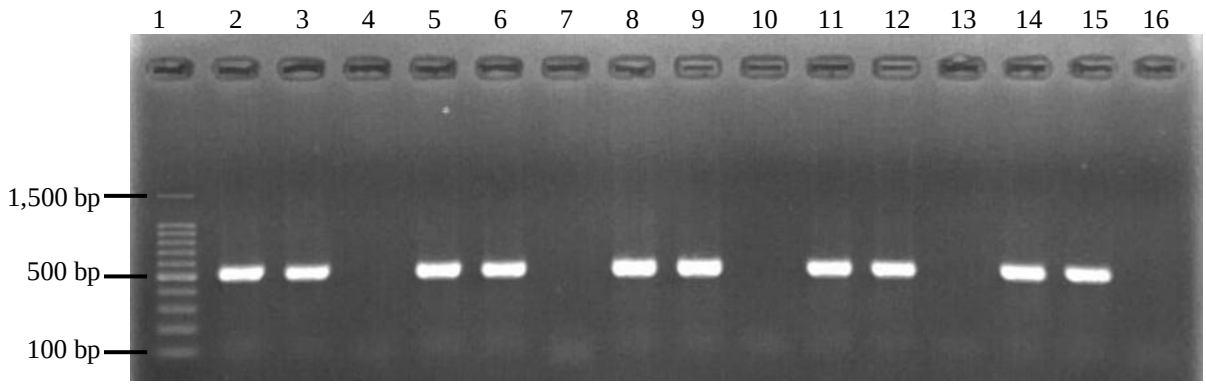

Figure 1: Electrophoresis analysis of pig-specific Polymerase Chain Reaction (PCR) products (522 bps) from PCR amplification at different temperatures. Lane 1: DNA Ladder 100 bp; lane 2-4: 46 °C; lane 5-7: 48 °C; lane 8-10: 50 °C; lane 11-13: 52 °C; 14-16: lane 54 °C; lane 4,7,10, 13, and 16: no template control.

[Downloaded from jfqhc.ssu.ac.ir on 2025-05-20]

[DOI: 10.18502/jfqhc.8.4.8256]

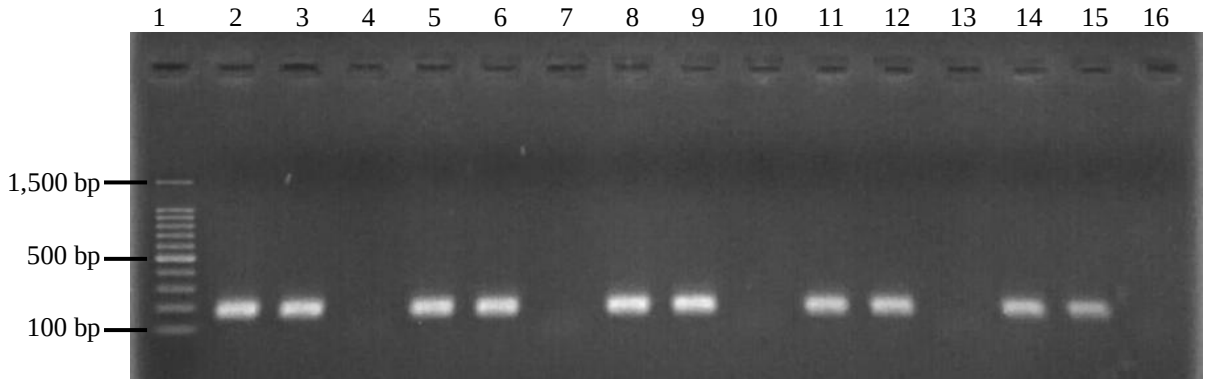

Figure 2: Electrophoresis analysis of cattle-specific Polymerase Chain Reaction (PCR) products (179 bps) from PCR amplification at different temperatures. Lane 1: DNA Ladder 100 bp; lane 2-4: 46 °C; lane 5-7: 48 °C; lane 8-10: 50 °C; lane 11-13: 52 °C; 14-16: lane 54 °C; lane 4,7,10, 13, and 16: no template control.

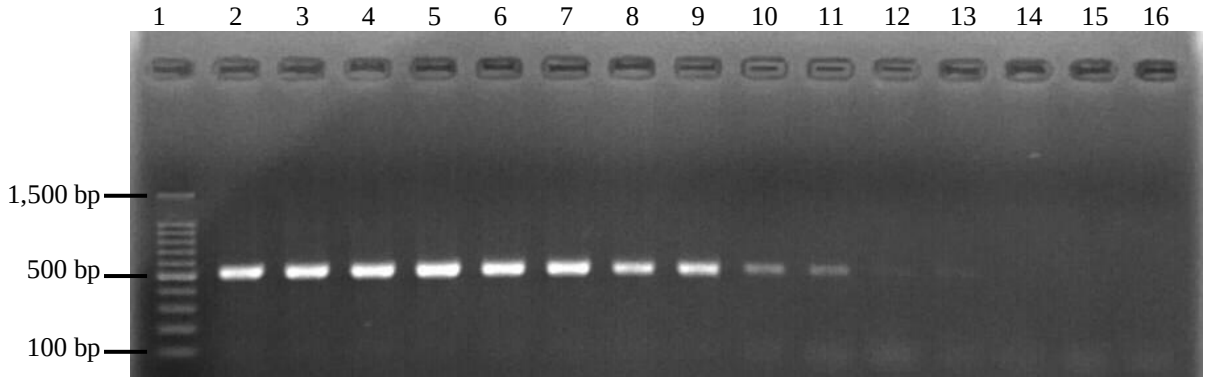

Figure 3: Electrophoresis analysis of pig-specific Polymerase Chain Reaction (PCR) products (522 bps) from PCR amplification at different template DNA concentration. Lane 1: DNA Ladder 100 bp; lane 2-3: 10 ng/µl; lane 4-5: 1 ng/µl; lane 6-7: 0.1 ng/µl; lane 8-9: 0.01 ng/µl; lane 10-11: 0.001 ng/µl; lane 12-13: 0.0001 ng/µl; lane 14-15: 0.00001 ng/µl; lane 16: no template control.

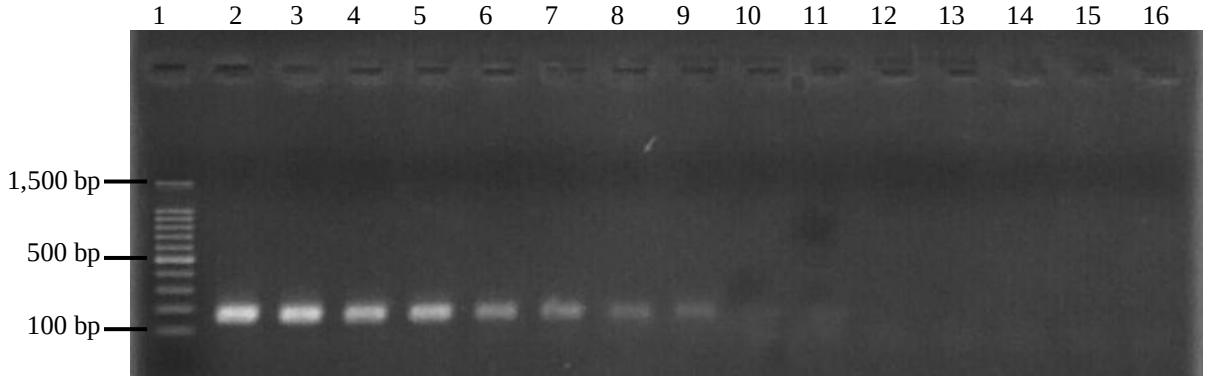

Figure 4: Electrophoresis analysis of cattle-specific Polymerase Chain Reaction (PCR) products (179 bps) from PCR amplification at different template DNA concentration. Lane 1: DNA Ladder 100 bp; lane 2-3: 10 ng/µl; lane 4-5: 1 ng/µl; lane 6-7: 0.1 ng/µl; lane 8-9: 0.01 ng/µl; lane 10-11: 0.001 ng/µl; lane 12-13: 0.0001 ng/µl; lane 14-15: 0.00001 ng/µl; lane 16: no template control.

[Downloaded from jfqhc.ssu.ac.ir on 2025-05-20]

[DOI: 10.18502/jfqhc.8.4.8256]

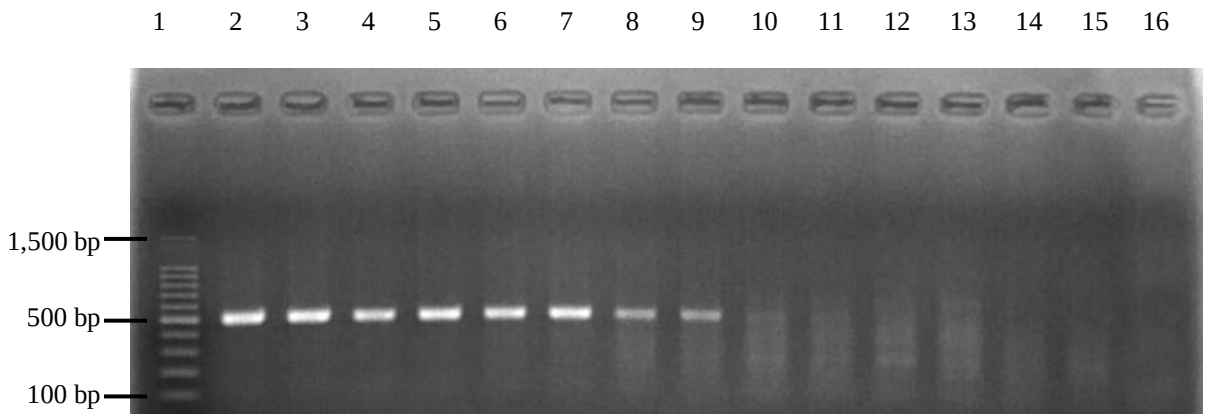

Figure 5: Electrophoresis analysis of Polymerase Chain Reaction (PCR) products (522 bps) from PCR amplification of DNA admixture of nine chordate species spiked with different concentrations of pig DNA. Lane 1: DNA Ladder 100 bp; lane 2-15: 200 ng/µl DNA admixture of nine chordate species spiked with lane 2-3: 10 ng/µl; lane 4-5: 1 ng/µl; lane 6-7: 0.1 ng/µl; lane 8-9: 0.01 ng/µl; lane 10-11: 0.001 ng/µl; lane 12-13: 0.0001 ng/µl; lane 14-15: not spiked; lane 16: no template control.

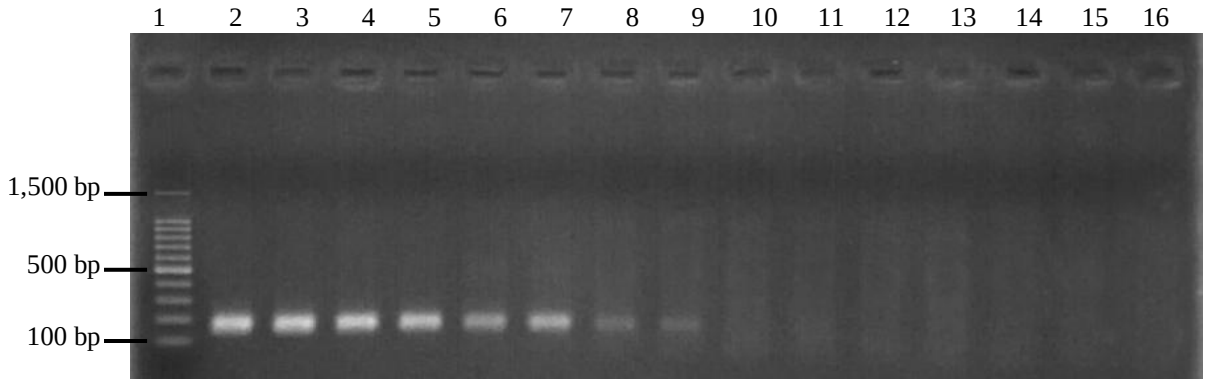

Figure 6: Electrophoresis analysis of Polymerase Chain Reaction (PCR) products (179 bps) from PCR amplification of DNA admixture of nine chordate species spiked with different concentrations of cattle DNA. Lane 1: DNA Ladder 100 bp; lane 2-15: 200 ng/µl DNA admixture of nine chordate species spiked with lane 2-3: 10 ng/µl; lane 4-5: 1 ng/µl; lane 6-7: 0.1 ng/µl; lane 8-9: 0.01 ng/µl; lane 10-11: 0.001 ng/µl; lane 12-13: 0.0001 ng/µl; lane 14-15: not spiked; lane 16: no template control.

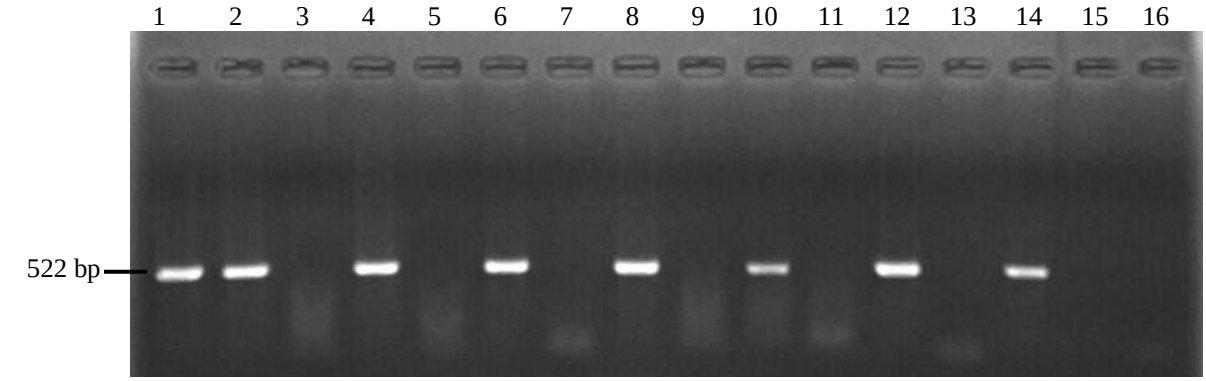

Figure 7: Electrophoresis analysis of Polymerase Chain Reaction (PCR) products (522 bps) from PCR amplification of industrially processed samples. Lane 1: positive control; lane 2-3: meat and bone meal-1 (MBM-1); lane 4-5: meat and bone meal - 2 (MBM-2); lane 6-7: crushed bones; lane 8-9: fish meal; lane 10-11: poultry meal; lane 12-13: vegetable fat; lane 14-15: milk cream; lane 2, 4, 6, 8, 10, 12, and 14: spiked with 1 µl pig DNA (10 ng/µl); lane 16: no template control.

[Downloaded from jfqhc.ssu.ac.ir on 2025-05-20]

[DOI: 10.18502/jfqhc.8.4.8256]

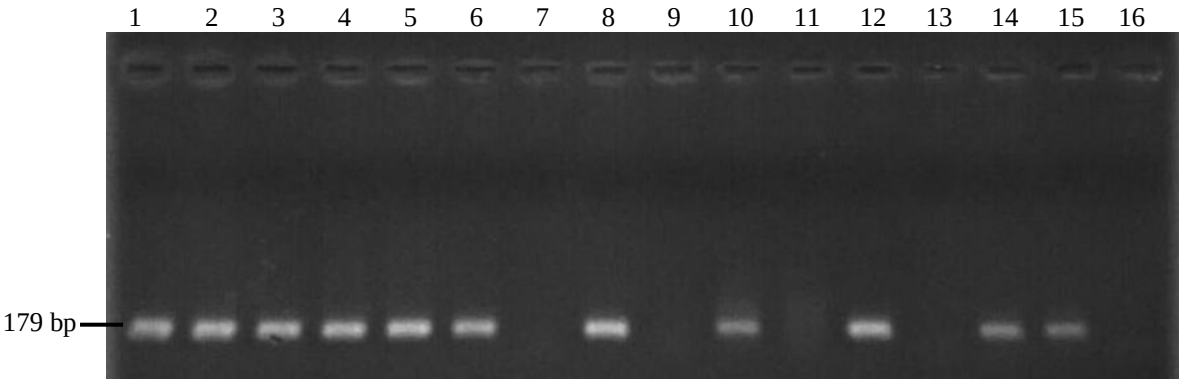

Figure 8: Electrophoresis analysis of Polymerase Chain Reaction (PCR) products (179 bps) from PCR amplification of industrially processed samples. Lane 1: positive control; lane 2-3: meat and bone meal-1 (MBM-1); lane 4-5: meat and bone meal - 2 (MBM-2); lane 6-7: crushed bones; lane 8-9: fish meal; lane 10-11: poultry meal; lane 12-13: vegetable fat; lane 14-15: milk cream; lane 2, 4, 6, 8, 10, 12, and 14: spiked with 1 µl cattle DNA (10 ng/µl); lane 16: no template control.

Discussion

Primers are one of the basic components of PCR. During filtering, primers are usually verified either to pass or not on the standard range, and limits for the examined constraints are defined. Because, the standards like length and matched or mismatched sequence of primers are crucial for producing PCR products with specificity and efficiency (De Melo et al., 2021). The specificity of PCR amplification reactions is usually established by the appropriate hybridization of primer specific sequences to complementary sequences by adjusting the annealing temperature (T_a), which is typically fixed between 40 and 65 °C (Lorenz, 2012). The annealing of primers is a critical step in PCR, and the annealing temperature influences the specificity of primer annealing (Starčič Erjavec, 2019). Furthermore, the rate of primer annealing to the template DNA is influenced by primer length and the efficiency of the PCR may be lowered if the primer length is too lengthy. As a result, primers with more than 30 base pairs are hardly used (Analytical Methods Committee, AMCTB No 59, 2014). In this experiment, no non-specific amplification was detected at all annealing temperature (46-54 °C) showing that the newly designed primers of 20-22 base pairs are highly specific to the 12S rRNA gene sequences. The species-specific amplification at 50 °C produced a band with the highest intensity (Figures 1 and 2) since the GC contents were less than 40%. During primer design, the GC content below the PCR standards (Analytical Methods Committee, AMCTB No 59, 2014) was directed based on the availability of the most identical regions of the 12S rRNA gene sequences. Thus, 50 °C was determined as the annealing temperature for subsequent PCR assay employing the both pig and cattle-specific primers.

In addition to annealing temperature, a number of variables can affect sensitivity, such as DNA consistency, primer specificity, etc. A PCR's sensitivity relies on the effective amplification of a minimum quantity of template DNA in a sample. As DNA isolation from various tissues can vary, the sensitivity of a PCR assay can therefore be best measured by quantifying the minimum amount of template DNA from which effective amplification is obtained (Vaithiyanathan and Kulkarni, 2016). In this experiment, at optimized annealing temperature (50 °C) PCR products of 0.001 ng total pig DNA (Figure 3) and 0.01 ng total cattle DNA (Figure 4) produced enough emission to be visible in ethidium bromide stained agarose gel. The variation in sensitivity may be due to variation of targeted mtDNA extraction. As only approximately 0.2% of total DNA is expected to be mitochondrial (Bosworth et al., 2017), the extracted cattle mtDNA might be lower than pig mtDNA. Because, the number of copies of mtDNA in various cell types varies greatly. Cells that usually need a lot of ATP from oxidative phosphorylation have a lot of copies of mtDNA, while cells that don't need as much have few copies (Lee et al., 2015; Picard, 2021; Torres, 2018). The sensitivity of pig-specific primers was found similar with the study of Che Man et al. (2012), but the sensitivity of cattle-specific was much lower in this study than the assay produced by Karabasanavar et al. 2017 that achieved 0.0001 ng target DNA sensitivity of raw meats.

Targeting multi-copy mitochondrial genes the species-

[Downloaded from jfqhc.ssu.ac.ir on 2025-05-20]

[DOI: 10.18502/jfqhc.8.4.8256]

specific PCR has gained considerable interest in recent years (Ali et al., 2012; Galal-Khallaf, 2021; Hossain et al., 2017). The detailed knowledge of the organization of animal mtDNA genes, along with the vast abundance of reported sequences of many animals, makes it possible to effectively design species-specific amplification primers. Besides, the intraspecific heterogeneity of mtDNA helps in determining the possibility of breed discrimination (Amaral et al., 2015). In this study, two primer pairs were specifically designed to detect the mitochondrial 12S rRNA gene sequence of pig and cattle. By precisely amplifying as little as ≥0.01 ng of pig or cattle DNA in admixture, each species-specific primer pair produced predicted PCR products of 522 and 179 bps only for pig and cattle respectively and not amplified the DNA of other animal species (Figures 5 and 6). The primer sequences mismatched with the mitochondrial 12S rRNA gene sequences of bat, bata fish, chicken, human, parrot, rat, sheep, toad, and pig/cattle and caused the primer to be unable to bind and amplify. This confirms that the newly designed primers are unique to pigs or cattle. Most investigations use 12S rRNA gene-based primers for sequencing or RFLP analysis for species identification, which necessitates the use of additional instruments as well as reagents and chemicals (Islam et al., 2021; Jadav et al., 2014). Again, according to the primer-BLAST result, the poorly designed pig and cattle-specific primers from 12S rRNA (Cahyadi et al., 2018) and cytochrome oxidase I (Wang et al., 2019) gene can recognize the respective complementary gene sequences along with the cattle or pig species like sheep, camel, horse, buffalo, and so on. As a consequence, false positive findings might be generated, causing havoc in the food and feed processing industries. Thus, the virtually and practically verified pig and cattle-specific primers under this study are more reliable and cost effective than the poorly designed primers of earlier studies.

Compounds such as maillard reaction products, milk proteins, fat, glycogen, collagen, iron, cobalt, etc. can also be transferred during the extraction of DNA from meat products to extracted DNA specimens. These compounds hinder the amplification of DNA and can have a significant negative effect on the research outcome (Spychaj et al., 2016). The ability of the various kits to free DNA from PCR inhibitors present in the samples is likely to depend on effective amplification (Vaithiyanathan and Kulkarni, 2016). Under this analysis the precisely amplified samples spiked with pig and cattle DNA denote that the samples did not contain any significant PCR inhibitory materials (Figures 7 and 8). Applying the primers in industrially processed food and feed sample analysis no amplification was observed for pig-specific primers; indicates absence of pig origin/derivatives (Figure 7) which was cross checked using the primers designed by Spychaj et al. (2016). But PCR amplification by cattle-specific primers of 3 samples implies that the samples contained the cattle origin/derivatives (Figure 8). Presence of cattle origin in the 3 samples indicates that the DNA of the samples was still not degraded though processed. Again, as neither pig nor cattle-specific primers did amplify the remaining 4 samples DNA, the samples having unamplified DNA must contain the origin other than pig or cattle. Moreover, as the analyzed results of industrially processed samples were similar to previously analyzed using the primers of Spychaj et al. (2016), the newly designed primer pairs are also specific to pig or cattle. However, practically an unknown sample may be composed of more than one species and may contain a very lower amount of target species or very lower amount of target DNA may be extracted as DNA admixture. Too low or the absence of target copy numbers in DNA admixture at the start of PCR amplification may greatly increase the probability of amplifying non-specific products. But competition of primers annealing to the sufficient target DNA sequences reduces the probability of amplifying non-specific products (Kalle et al., 2014). We suggest that sufficient copies of the target sequence should ideally be present at the start of PCR amplification.

Conclusion

This work effectively established a pig and cattle-specific PCR test based on the mitochondrial 12S rRNA gene sequence for the identification of pig and cattle in food and feed products, whether pure or mixed. The only recognition of the target mitochondrial gene sequence of pig and cattle species ensured that the respective set of primers were more unique than the earlier 12S rRNA or cytochrome oxidase I gene-based primers. The results showed that using the newly designed pig and cattle-specific primers is a safe and repeatable. As a result, the approach established in this work will be a useful tool for identifying pig or cattle species in food and feed items.

Author contributions

D.C.R. conceived and designed research and prepared original draft; D.C.R., S.A., C.L., and D.I. conducted the experiments; A.K.S., L.C.M., and M.A.A.K. supervised, investigated, and reviewed the manuscript; M.M.K.H. validated the results. All authors read and approved the final manuscript.

Conflicts of interest

There are no conflicts of interest.

[Downloaded from jfqhc.ssu.ac.ir on 2025-05-20]

[DOI: 10.18502/jfqhc.8.4.8256]

Acknowledgements

We are expressing our sincere and utmost gratitude to the Biomedical and Toxicological Research Institute (BTRI), BCSIR, Dhaka, Bangladesh for both the financial and logistic support. We also thank all the importers and retailers who provided us the samples for testing. This work was funded by the Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka, Bangladesh.

References

Ali M.E., Kashif M., Uddin K., Hashim U., Mustafa S., Che Man Y.B. (2012). Species authentication methods in foods and feeds: the present, past, and future of halal forensics. *Food Analytical Methods*. 5: 935–955. [DOI: 10.1007/s12161-011-9357-3]

Amaral J.S., Santos C.G., Melo V.S., Costa J., Oliveira M.B.P.P., Mafra I. (2015). Identification of duck, partridge, pheasant, quail, chicken and turkey meats by species-specific PCR assays to assess the authenticity of traditional game meat Alheira sausages. *Food Control*. 47: 190–195. [DOI: 10.1016/j.foodcont.2014.07.009]

Amorim A., Fernandes T., Taveira N. (2019). Mitochondrial DNA in human identification: a review. *PeerJ*. 7: e7314. [DOI: 10.7717/peerj.7314]

Analytical Methods Committee, AMCTB No 59. (2014). PCR - the polymerase chain reaction. *Analytical Methods*. 6: 333–336. [DOI: 10.1039/C3AY90101G]

Barakat H., El-Garhy H.A.S., Moustafa M.M.A. (2014). Detection of pork adulteration in processed meat by species-specific PCR-QIAxcel procedure based on D-loop and *cytb* genes. *Applied Microbiology and Biotechnology*. 98: 9805–9816. [DOI: 10.1007/s00253-014-6084-x]

Bosworth C.M., Grandhi S., Gould M.P., LaFramboise T. (2017). Detection and quantification of mitochondrial DNA deletions from next-generation sequence data. *BMC Bioinformatics*. 18: 407. [DOI: 10.1186/s12859-017-1821-7]

Cahyadi M., Puruhita., Barido F.H., Hertanto B.S. (2018). Specific primer design of mitochondrial 12S rRNA for species identification in raw meats. *IOP Conference Series: Earth and Environmental Science*. 102: 012038. [DOI: 10.1088/1755-1315/102/1/012038]

Che Man Y.B., Mustafa S., Khairil Mokhtar N.F., Nordin R., Sazili A.Q. (2012). Porcine-specific polymerase chain reaction assay based on mitochondrial D-loop gene for identification of pork in raw meat. *International Journal of Food Properties*. 15: 134–144. [DOI: 10.1080/10942911003754692]

Cho A.R., Dong H.J., Cho S. (2014). Meat species identification using loop-mediated isothermal amplification assay targeting species-specific mitochondrial DNA. *Korean Journal for Food Science of Animal Resources*. 34: 799–807. [DOI: 10.5851/kosfa.2014.34.6.799]

Costassa E.V., Iulini B., Mazza M., Acutis P., Maurella C., Meloni D., Pautasso A., Capucci L., Bozzetta E., Simmons M.M., Zanusso G., Pocchiari M., et al. (2016). Pathogenesis and transmission of classical and atypical BSE in cattle. *Food Safety*. 4: 130–134. [DOI: 10.14252/foodsafetyfscj.2016018]

De Melo A.A., Nunes R., Telles M.P.D.C. (2021). Same information, new applications: revisiting primers for the avian COI gene and improving DNA barcoding identification. *Organisms Diversity and Evolution*. 21: 599–614. [DOI: 10.1007/s13127-021-00507-x]

Di Pinto A., Bottaro M., Bonerba E., Bozzo G., Ceci E., Marchetti P., Mottola A., Tantillo G. (2015). Occurrence of mislabeling in meat products using DNA-based assay. *Journal of Food Science and Technology*. 52: 2479–2484. [DOI: 10.1007/s13197-014-1552-y]

Fajardo V., González I., Rojas M., García T., Martín R. (2010). A review of current PCR-based methodologies for the authentication of meats from game animal species. *Trends in Food Science and Technology*. 21: 408–421. [DOI: 10.1016/j.tifs.2010.06.002]

Fang X., Zhang C. (2016). Detection of adulterated murine components in meat products by TaqMan© real-time PCR. *Food Chemistry*. 192: 485–490. [DOI: 10.1016/j.foodchem.2015.07.020]

Galal-Khallaf A. (2021). Multiplex PCR and 12S rRNA gene sequencing for detection of meat adulteration: a case study in the Egyptian markets. *Gene*. 764: 145062. [DOI: 10.1016/j.gene.2020.145062]

Hossain M.A.M., Ali M.E., Hamid S.B.A., Asing., Mustafa S., Desa M.N.M., Zaidul I.S.M. (2017). Targeting double genes in multiplex PCR for discriminating bovine, buffalo and porcine materials in food chain. *Food Control*. 73: 175–184. [DOI: 10.1016/j.foodcont.2016.08.008]

Hutasoit I., Ramadhani Munthe F., Wiyah M., Nasution H., Pranata A.W. (2021). Real-time PCR to identify porcine DNA in prosthodontic materials. *Cumhuriyet Dental Journal*. 24: 216–223. [DOI: 10.7126/cumudj.887101]

Islam A., Halder J., Rahman A.M., Ud-Daula A., Uddin S., Hossain M.K., Jahan N., Alim A., Bhuyan A.A., Rubaya., Hasan M., Alam J. (2021). Meat origin differentiation by polymerase chain reaction-restriction fragment length polymorphism. *International Journal of Food Properties*. 24: 1022–1033. [DOI: 10.1080/10942912.2021.1953068]

Izadpanah M., Mohebali N., Elyasi Gorji Z., Farzaneh P., Vakhshiteh F., Shahzadeh Fazeli S.A. (2018). Simple and fast multiplex PCR method for detection of species origin in meat products. *Journal of Food Science and Technology*. 55: 698–703. [DOI: 10.1007/s13197-017-2980-2]

Jadav K., Rajput N., Shrivastav A.B., Mandal S., Shrivastav G. (2014). Application of 12S rRNA gene sequence for identification of Indian wild pig (*Sus scrofa cristatus*). *Journal of Meat Science and Technology*. 2: 79–84

Johnson R. (2014). Food fraud and "economically motivated adulteration" of food and food ingredients. *Congressional Research Service*. Washington D.C.

Kalle E., Kubista M., Rensing C. (2014). Multi-template polymerase chain reaction. *Biomolecular Detection and Quantification*. 2: 11–29. [DOI: 10.1016/j.bdq.2014.11.002]

Karabasanavar N., Girish P.S., Kumar D., Singh S.P. (2017). Detection of beef adulteration by mitochondrial D-loop based species-specific polymerase chain reaction. *International Journal of Food Properties*. 20: 2264–2271. [DOI: 10.1080/10942912.2017.1369103]

Khanzadeh F., Khaghaninia S., Maleki-Ravasan N., Koosha M., Oshaghi M.A. (2020). Molecular detection of *Dirofilaria* spp. and host blood-meal identification in the *Simulium turgaicum* complex (Diptera: Simuliidae) in the Aras River Basin, northwestern Iran. *Parasites and Vectors*. 13: 548. [DOI: 10.1186/s13071-020-04432-4]

Kumar A., Kumar R.R., Sharma B.D., Gokulakrishnan P., Mendiratta S.K., Sharma D. (2015). Identification of species origin of meat and meat products on the DNA basis: a review. *Critical Reviews in Food Science and Nutrition*. 55: 1340-1351. [DOI: 10.1080/10408398.2012.693978]

Lee W., Johnson J., Gough D.J., Donoghue J., Cagnone G.L.M., Vaghjiani V., Brown K.A., Johns T.G., St. John J.C. (2015). Mitochondrial DNA copy number is regulated by DNA methylation and demethylation of *POLGA* in stem and cancer cells and their differentiated progeny. *Cell Death and Disease*. 6: e1664. [DOI: 10.1038/cddis.2015.34]

Lorenz T.C. (2012). Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. *Journal of Visualized Experiments*. 63: e3998. [DOI: 10.3791/3998]

Nehal N., Choudhary B., Nagpure A., Gupta R.K. (2021). DNA barcoding: a modern age tool for detection of adulteration in food. *Critical Reviews in Biotechnology*. 41: 767-791. [DOI: 10.1080/07388551.2021.1874279]

Picard M. (2021). Blood mitochondrial DNA copy number: what

[Downloaded from jfqhc.ssu.ac.ir on 2025-05-20]

[DOI: 10.18502/jfqhc.8.4.8256]

are we counting? *Mitochondrion*. 60: 1–11. [DOI: 10.1016/j.mito.2021.06.010]

Ricardo P.C., Françoso E., Arias M.C. (2020). Mitochondrial DNA intra-individual variation in a bumblebee species: a challenge for evolutionary studies and molecular identification. *Mitochondrion*. 53: 243–254. [DOI: 10.1016/j.mito.2020.06.007]

Rojas M., González I., Pavón M.Á., Pegels N., Hernández P.E., García T., Martín R. (2011). Application of a real-time PCR assay for the detection of ostrich (*Struthio camelus*) mislabelling in meat products from the retail market. *Food Control*. 22: 523–531. [DOI: 10.1016/j.foodcont.2010.09.039]

Spychaj A., Szalata M., Słomski R., Pospiech E. (2016). Identification of bovine, pig and duck meat species in mixtures and in meat products on the basis of the mtDNA cytochrome oxidase subunit I (*COI*) gene sequence. *Polish Journal of Food and Nutrition Sciences*. 66: 31–36. [DOI: 10.1515/pjfns-2015-0051]

Starčič Erjavec M. (2019). Annealing temperature of 55°C and specificity of primer binding in PCR reactions. *Synthetic Biology-New Interdisciplinary Science*. [DOI: 10.5772/intechopen.85164]

Tibola C.S., Da Silva S.A., Dossa A.A., Patrício D.I. (2018). Economically motivated food fraud and adulteration in Brazil: incidents and alternatives to minimize occurrence. *Journal of Food Science*. 83: 2028–2038. [DOI: 10.1111/1750-3841.14279]

Torres J.A.A. (2018). The mitochondrial DNA copy number used as biomarker. *International Journal of Molecular Biology*. 3: 115-117. [DOI: 10.15406/ijmboa.2018.03.00063]

Vaithiyanathan S., Kulkarni V.V. (2016). Species identification of cattle and buffalo fat through PCR assay. *Journal of Food Science and Technology*. 53: 2077–2082. [DOI: 10.1007/s13197-016-2198-8]

Wang L., Hang X., Geng R. (2019). Molecular detection of adulteration in commercial buffalo meat products by multiplex PCR assay. *Food Science and Technology*. 39: 344–348. [DOI: 10.1590/fst.28717]

Zhang X., Armani A., Giusti A., Wen J., Fan S., Ying X. (2021). Molecular authentication of crocodile dried food products (meat and feet) and skin sold on the Chinese market: implication for the European market in the light of the new legislation on reptile meat. *Food Control*. 124: 107884. [DOI: 10.1016/j.foodcont.2021.107884]

[Downloaded from jfqhc.ssu.ac.ir on 2025-05-20]

[DOI: 10.18502/jfqhc.8.4.8256]