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Validating duplex-PCR targeting *ND2* for bovine and porcine detection in meat products

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ABSTRACT

Food authentication is a mandatory effort to assure the fair-trade. This study developed a duplex polymerase chain reaction (PCR) from the NADH dehydrogenase subunit 2 (*ND2*) gene to amplify specific segments of a cattle and porcine DNA. A universal forward primer composed of nineteen base pairs (bp) (3′-CCAAACA-CAACTCCGAAAA-5′) and species-specific reverse primers composed of twenty (3′-CCAAACACAACTCCGAAAA-5′) and twenty-one (3′-TGGCAAGAATTAGGACGGTTA-5′) bp were used to limit the amplified DNA segment for porcine and cattle. The PCR reaction would generate a product with a profile of 168 and 227 bp, respectively. To investigate the accuracy and limit of detection, an *in vitro* experiment was conducted using simplex and duplex PCR on commercial meatballs randomly purchased from a commercial market in Surakarta, Indonesia. The findings of this study indicated that *ND2* could be used as an alternative genetic marker for the identification of porcine and beef species in meat-derived products.

1. Introduction

Economically motivated adulteration remains an unresolved problem in retail markets. It encompasses the adulteration of meat products as a food product category that is most frequently faked through the substitution of meat products with undeclared lower value meats (Wibowo et al., 2023). Supported by the product policy law issued by the European Commission (2001), labeling authentication must provide the detailed information required by consumers as a guarantee of the products they consume (Soares et al., 2010), because consumers' decisions to buy meat products are mainly motivated by lifestyle, religious limitations, cultural influences, and fairtrade practices (Kesmen et al., 2013).

To date, adulteration of meat products is a widespread issue that has not been properly addressed in the Indonesian market. A study by Ali et al. (2012) revealed that porcine contamination is frequently identified in many processed beef products in many Indonesian metropolitan cities. One of the main requirements for meat products that can be widely marketed in Indonesia is that they meet the halal criteria, which must surpass strict hygienic and fulfill complex religious and health indictments. This creates a higher production cost for producers compared to that of conventional meat products and opens the opportunity for fraud by suppressing the production cost to gain optimum income in non-permissible ways (Fajardo et al., 2010; Ali et al., 2012).

In addition to biomedicine and genomic mapping studies, polymerase chain reaction (PCR) is used in food-related areas to identify species-specific origins of food (Girish et al., 2013). To date, food researchers have utilized both genomic and mitochondrial genetic markers to reveal the species information contained within samples (Cahyadi et al., 2018). Through multiplex PCR technique, Uddin et al. (2021) accurately discriminated and identified beef, buffalo, chicken, duck, goat, sheep, and porcine DNA in meat samples. Additionally, an extended study revealed that this approach could be effectively used to identify diverse species of dogs and rats contained within beef batter (Cahyadi et al., 2019). PCR-based techniques harness the genetic materials that exist within DNA and offer a wide range of options for exploring unique structural sequences. The uniqueness of each DNA makes molecular PCR techniques efficient for meat authentication up to species level

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(Boyrusbianto et al., 2023). In comparison to meat authentication methods using protein biomarkers that show fragility and structural modifications throughout the processing stages (Ali et al., 2012), DNA-based authentication accentuates greater survival and resilience due to the unique circular sequence, high copy number, and shorter length, enabling accurate quantification from extremely processed meat mixtures (Ni'mah et al., 2016; Novianty et al., 2017). As a result, molecular PCR techniques have emerged as a practical, accurate, and reliable method for meat authentication, even for samples containing limited DNA fragments (Safdar and Junejo, 2016).

However, the successful amplification of DNA fragments by PCR largely relies on the sequence and specificity of oligonucleotide molecules, or short single strands of DNA, called primers (Li et al., 2019; Kang, 2019). Along with the DNA template, primers are another important PCR component that serve as a specific target limiter of the DNA template (Kang, 2019). The principle of designing ideal primers is based on the fact that some chromosomes may share a similarity in nucleotide sequences through homologous regions or unintentional matches; therefore, such a shared region can be utilized to design the primer and mediate its binding to the targeted DNA fragment. Additionally, primers must comply with the suggested length of 18–24 bp and have at least 40–60% composition of guanine/cytosine (G/C) with the absence of 3 or >G and C content at the 3′-end (Bustin et al., 2020).

NADH dehydrogenase subunit 2 (*ND2*) is a member of the mitochondrial DNA (mtDNA) family. Its protein product is a 39 kDa protein with 347 amino acids. *ND2* is involved in the essential respiratory complex by assembling the core proteins required to catalyze NADH dehydrogenase (Attardi and Schatz, 1988). Evolutionary studies have suggested that this gene possesses more variability than other genetic markers such as Cyt-b and 12S rRNA (Mohamadzade Namin et al., 2022). A higher level of variability results in a faster rate of molecular evolution of the ND2 sequence, resulting in significantly different sequences among species (Hamilton, 2001; Kocher et al., 1995; Mohamadzade Namin et al., 2022). To the best of our knowledge, no information is available regarding the use of *ND2* as a genetic marker in primer design for the quantitative detection of meat adulteration. Furthermore, limit of detection (LOD) that commonly at no more than 0.1 ng/μL (Luo et al., 2008; Gupta et al., 2012) needs more exploration. A lower LOD would allow food authentication on meat products that have surpassed an extreme processing stages, assuring its reliability. Therefore, the purpose of this study was to evaluate the reliability of primers designed using *ND2* as a genetic marker for the authentication of meat products.

2. Materials and methods

2.1. Primer design

Fragment DNA sequences of the *ND2* gene from the porcine and cattle species were obtained from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov). The accession numbers of the species used in this study were AB292606.1 and AF492350.1 for porcine and beef, respectively. Sequence data in FASTA form, a text-based format for the representation of nucleotide or amino acid sequences, was then input into the PRIMER3^{TM} online software (https://bioinfo.ut.ee/primer3-0.4.0/). Subsequently, the alignment analysis was performed to determine the consensus or a unique area for specific binding of the obtained primers, avoiding attachment to the unintended areas of other organisms (Cahyadi et al., 2018). This area contained limited or no mutations.

Alignment was confirmed through BLAST analysis (https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi). Alignment analysis revealed a degree of similarity among species in a specific *ND2* fragment, particularly for pigs and cattle (Crossley et al., 2020). Primer candidates were generated by inputting the FASTA sequence of the *ND2* gene for each species into Primer3 Plus online software (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cg). This software assists users in providing primer candidates with various properties, including primer size, primer position, GC composition, and amplicons (Hung and Weng, 2016). The selected forward and reverse primers were subsequently examined on the target sequence based on the order of the codons.

2.2. DNA extraction

Fresh *Longissimus lumborum* from pork and beef samples were purchased randomly from a traditional market in Surakarta, Central Java, Indonesia (October 2022). They were ground directly using different meat grinders (Meat Mincer LH-22CW; Huamei, Zhejiang, China) to avoid cross-contamination. Ground meat samples were randomly allocated into five different groups: simplex PCR-porcine (S1), simplex PCR-beef (S2), duplex-PCR porcine (D1), duplex-PCR beef (D2), and duplex PCR containing 50% porcine + 50% beef mixture (X), all the samples were prepared in triplicates. To evaluate the primers on the processed products, an *in vitro* assay was conducted on meatball products labeled as beef. They were randomly purchased from several traditional markets in Surakarta, Central Java, Indonesia. Each of samples were prepared in ten replications (n = 60).

Extraction was performed according to the method described by Cahyadi et al. (2018), with minor modifications. Briefly, the DNA genomes of porcine and beef samples were prepared according to a previously described protocol using the gsync^{TM} DNA extraction kit for animal tissues (Geneaid Biotech Ltd., New Taipei City, Taiwan). A total of 30 mg of meat tissue from each porcine and beef sample was transferred into a 1.5 mL microcentrifuge tube and ground. A certain amount of 200 μL GT buffer was added and homogenized. Twenty microliters of proteinase K (Geneaid Biotech Ltd., New Taipei City, Taiwan) was added to the mixture and incubated at 60 °C overnight until a clear lysate was visible.

Following overnight incubation, the supernatant was transferred into a new microtube with the subsequent addition of 200 μL of GSB buffer (Geneaid Biotech Ltd., New Taipei City, Taiwan) and vortexed for 10 s. Absolute ethanol was added and homogenized. The mixture was then placed into a GS column, which was paired with a 2 mL collection tube, centrifuged at 14,000–16,000 rpm for 1 min, and the 2 mL collection tube was then replaced with a new tube. Afterward, 400 μL of W1 buffer was added to the GS column and purified for another 30 s. The GS column was then put into the other collection tube and mixed with 600 μL of wash buffer, centrifuged for 3 min, and transferred to a 1.5 mL microtube with the addition of 200 μL previously incubated elution buffer. The mixtures were allowed to stand for 3 min and centrifuged for 30 s to elute the DNA. The presence of DNA extract was confirmed by electrophoresis on a 1% agarose gel and visualized via gel document (Glite UV, Pacific Image, Taiwan).

2.3. Running PCR

Running and optimalization of PCR technique were performed according to the method described by Cahyadi et al. (2018) with minor modifications. Briefly, a total of 25 μL solutions were prepared, consisting of 12.5 μL from the 2X KAPA2G Fast Multiplex kit (KAPA Biosystems. Inc., Massachusetts, USA), 0.5 μL (10 μM) of both forward and reverse primers, 1 μL of porcine and beef DNA templates, and 8.5 μL aquabidest. PCR conditions were as follows: denaturation initiation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for another 15 s, annealing for 30 s at 60 °C, initial extension for 30 s at 72 °C and final extension for 3 min at 72 °C. To confirm the success of the PCR analysis, it was visualized through electrophoresis on a 2% agarose gel and observed through the gel document (Glite UV, Pacific Image, Taiwan).

2.4. Sequence analysis

Sequence analysis was performed to confirm that the PCR products specifically attached to the target DNA fragment. It also provided information on the degree of similarity of the PCR products obtained for each sequence to the references. Sequencing of PCR products generated from the *ND2* gene was conducted at PT. Genetics Science, Indonesia. The sequencing results were subsequently processed using BioEdit 7.2.6.1; the FASTA sequence was copied and aligned using Clustal Omega online software (https://www.ebi.ac.uk/Tools/msa/clustalo/).

3. Results and discussion

3.1. Primer design

This study proposes primer pairs designed from the consensus region of the *ND2* gene in pigs and cattle (Table 1). Two pairs of primers were designed using online software, with slight modifications considering conserved area. The primer pairs were composed of universal forward (UF) and reverse (R) primers to amplify both the pig and cattle species. The UF primer targeting the initial or starting sequence of the *ND2* gene of both pig and cattle species consisted of 20 bp (5′-CCAAACACAACTCCGAAAA-3′). Additionally, the R primers of twenty (3′-CCAAACACAACTCCGAAAA-5′) and twenty-one (3′-TGGCAAGAATTAGGACGGTTA-5′) nucleotide bp limited the end of sequence amplification from pig and cattle sequences, respectively. The PCR amplification product using primers from the *ND2* gene fragment in this study resulted in product sizes of 168 and 227 bp for pig and cattle species, respectively.

Careful selection of an ideal primer is an essential first step to successful DNA amplification using the PCR technique. Ideally, primers should exhibit high specificity and efficiency. Specificity refers to the frequency of mispriming errors, whereas efficiency refers to the ability of a primer to optimally amplify target DNA fragments two times better in each PCR cycle (Dieffenbach et al., 1993; Li et al., 2019; Bustin et al., 2020). The ideal primer design must accommodate the intra-species conserved area and inter-species polymorphisms to avoid mismatching and improve specificity (Ali et al., 2012). Additionally, the primer melting temperature (Tm) is highly affected by its nucleotide composition; therefore, the optimal length, composition of GC content, and existence of G and C content at the 3′-end should be taken into account when designing primer (Thornton and Basu, 2015). This is a critical stage prior to performing PCR because an ideal primer design determines the specificity and efficiency of the amplified DNA fragment in a single reaction (Ye et al., 2012).

3.2. Primer reliability

Simplex and duplex PCR were carried out to test and confirm the reliability of the primers in single and mixed sample environments, and the results are shown in Fig. 1. PCR results produced a clear band for all target samples with no additional fragments: simplex PCR for porcine (PS1) and cattle (CS2) samples; duplex PCR for porcine (PD1) and beef (CD2) samples with only a single band; and cross-species specificity by duplex PCR, resulting in clearly visible double bands representing the combination of porcine and beef samples (DPC). The reactions generated specific PCR product sizes: 168 BP for porcine samples and 227 BP for beef samples.

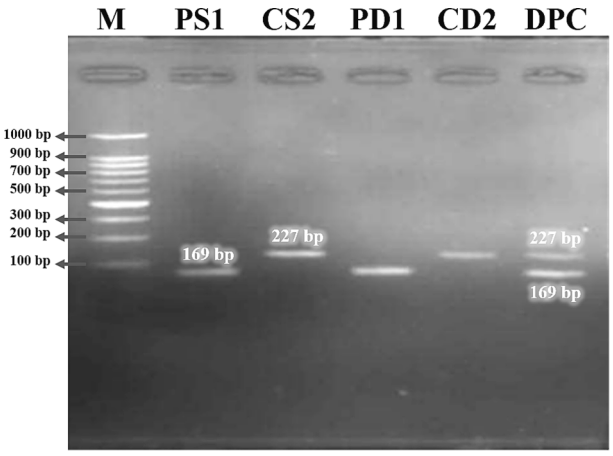

Fig. 1. Gel document visualization of simplex-PCR for the species detection in meat samples targeting cattle and porcine mtDNA *ND2*. M indicates 100 bp marker ladder; PS1 indicates simplex-PCR for porcine species; CS2 indicates simplex-PCR for cattle species; PD1 indicates duplex-PCR containing only porcine species; CD2 indicates duplex-PCR containing only cattle species; and DPC indicates duplex-PCR containing 50% porcine and 50% cattle species within the samples. 169 bp is the product size for porcine and 227 is the product size for cattle species in this study.

In terms of PCR reaction optimization, designed primers from *ND2* reached optimum amplification during one single PCR reaction at 63 °C. The simplex and duplex PCR reactions lasted for 30 cycles, with an initial denaturation step at 95 °C for 3 min, denaturation step at 95 °C for 15 s, annealing at 63 °C for 30 s, extension at 72 °C for another 30 s and the final extension at 72 °C for 3 min. Primer design accounts for several PCR parameters, particularly the melting and annealing temperatures, which are possibly calculated to be 5 °C lower than the actual Tm of a primer (Svec et al., 2015). Ideally designed primers that meet these requirements will enhance the specificity and limit of detection of meat products, even those that have undergone extreme processing stages (Ali et al., 2012).

Genetic markers used as references for primer design must satisfy the theoretical requirements for ideal primers. They must possess unique and high polymorphisms among organisms; therefore, they do not share a large number of similar genetic sequences. This is consistent with previous studies by Kocher et al. (1995) and Kalia et al. (2004), who suggested the utilization of *ND2* as a genetic marker because of its high degree of mutation, resulting in high variations. As information on *ND2* gene utilization as a genetic marker in food is limited, our study provided satisfactory results, which can be seen from its accuracy, specificity, and efficiency. This implied that the primer design generated from the *ND2* gene through the online software Primer3^{TM} had good reliability in limiting the target DNA fragment of each sample, without any mismatching.

Table 1
Primer candidate designed from *ND2 gene* for Cattle and Pig.

No.	Species	Primer		Length	Position	Product Size
1	Cattle	UF[1]	CCAAACACAACTCCGAAAA	20 bp	512–532	227 bp
		R[2]	TGGCAAGAATTAGGACGGTTA	21 bp	718–739	
2	Pig	UF[1]	CCAAACACAACTCCGAAAA	20 bp	512–532	168 bp
		R[2]	GTTGTGGTTGCTGAGCTGTG	20 bp	660–680	

[1] UF, Universal forward primer.
[2] R, Reverse primer.

3.3. Limit of detection (LOD)

The primer sensitivity was measured using the limit of detection (LOD). It states that the lowest concentration of DNA or RNA material needs to be attached and amplified using primers through PCR. Considering that the identification of fraudulent foods mostly involves products that undergo extreme cooking processes, the determination of LOD is essential to ensure that even the smallest sequence target concentration can be detected with high accuracy (Hossain et al., 2017). The LOD of the primers designed for the ND2 gene is presented in Figs. 2-4. In this study DNA templates extracted from both target species were serially diluted from higher to lower concentration ranged from 25 to 0.0001 ng/ μL. The serial dilution was done in accordance to that of previously published study by Ali et al. (2015).

Using simplex and duplex PCR, this study attempted to determine the limit of successful amplification of the *ND2* sequence from beef and porcine using the designed primers. The *ND2* primer LOD was qualitatively measured based on the BP intensity that was observed under gel visualization. The bright and clear intensity of the observed DNA band indicated a considerable LOD. Fig. 5A shows the LOD visualization for beef (S), with a product size of 227 BP. Various concentrations of the DNA template (S1:25 ng/μL; S2:10 ng/μL; S3:1 ng/μL; S4:0.1 ng/μL; S5:0.01 ng/μL; S6:0.001 ng/μL; S7:0.0001 ng/μL; and S:negative control) were compared and the results were finely recorded as LOD. Based on the result, the intensity of the well was still clearly seen until S7, indicating the identification of bovine species using *ND2 gene* is still possible even when the processed samples contain only 0.0001 ng/μL in DNA concentration. Similarly, among eight distinct bands observed under gel documentation for porcine species (5B), all well displayed clear visual appearance under gel documentation up to 0.0001 ng/μL. The LOD under simplex PCR was carefully performed to clarify whether primer would possess great accuracy and avoid cross-amplification against non-targeted species. With regard to the LOD duplex PCR, as seen in Fig. 5C, gel-view showed all investigated concentrations showed clear bands with the product size of 169 and 227 bp for porcine and beef, respectively. It confirms the LOD of this study using *ND2 gene* as genetic marker was at 0.0001 ng/μL DNA concentration. Ali et al. (2015)

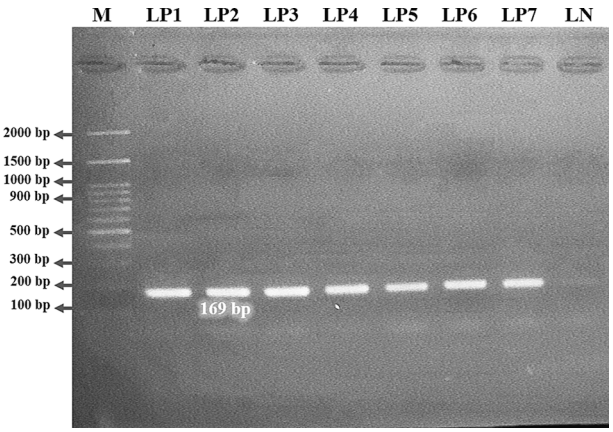

Fig. 2. Gel document visualization of limit of detection (LOD) for the species detection in meat samples targeting porcine mtDNA *ND2*. M indicates 100 bp marker ladder; LP1 indicates simplex-PCR containing porcine DNA at a concentration of 25 ng/μL; LP2 indicates simplex-PCR containing porcine DNA at a concentration of 10 ng/μL; LP3 indicates simplex-PCR containing porcine DNA at a concentration of 1 ng/μL; LP4 indicates simplex-PCR containing porcine DNA at a concentration of 0,1 ng/μL; LP5 indicates simplex-PCR containing porcine DNA at a concentration of 0,01 ng/μL; LP6 indicates simplex-PCR containing porcine DNA at a concentration of 0,001 ng/μL; LP7 indicates simplex-PCR containing porcine DNA at a concentration of 0,0001 ng/μL; LN indicates a negative control. 169 is the product size for porcine species in this study.

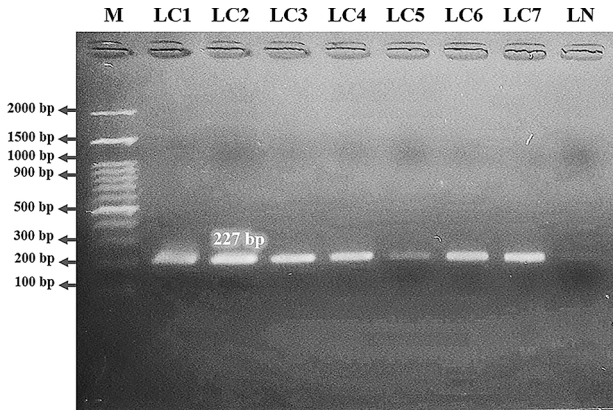

Fig. 3. Gel document visualization of limit of detection (LOD) for the species detection in meat samples targeting cattle mtDNA *ND2*. M indicates 100 bp marker ladder; LC1 indicates simplex-PCR containing cattle DNA at a concentration of 25 ng/μL; LC2 indicates simplex-PCR containing cattle DNA at a concentration of 10 ng/μL; LC3 indicates simplex-PCR containing cattle DNA at a concentration of 1 ng/μL; LC4 indicates simplex-PCR containing cattle DNA at a concentration of 0,1 ng/μL; LC5 indicates simplex-PCR containing cattle DNA at a concentration of 0,01 ng/μL; LC6 indicates simplex-PCR containing cattle DNA at a concentration of 0,001 ng/μL; LC7 indicates simplex-PCR containing cattle DNA at a concentration of 0,0001 ng/μL; LN indicates a negative control. 227 is the product size for cattle species in this study.

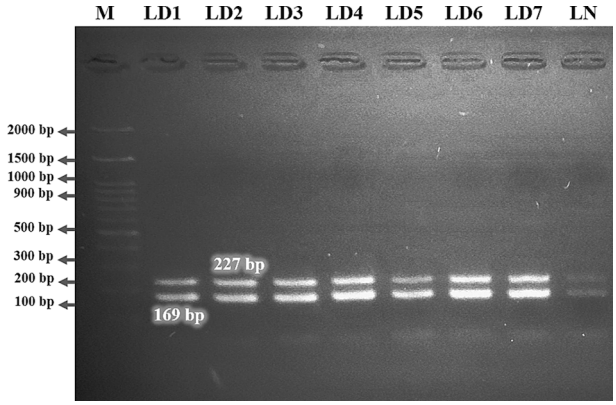

Fig. 4. Gel document visualization of limit of detection (LOD) for the species detection in meat samples targeting porcine and cattle mtDNA *ND2 gene*. M indicates 100 bp marker ladder; LD1 indicates duplex-PCR containing porcine and cattle DNA at a concentration of 25 ng/μL; LD2 indicates duplex-PCR containing porcine and cattle DNA at a concentration of 10 ng/μL; LD3 indicates duplex-PCR containing porcine and cattle DNA at a concentration of 1 ng/μL; LD4 indicates duplex-PCR containing porcine and cattle DNA at a concentration of 0,1 ng/μL; LD5 indicates duplex-PCR containing porcine and cattle DNA at a concentration of 0,01 ng/μL; LD6 indicates duplex-PCR containing porcine and cattle DNA at a concentration of 0,001 ng/μL; LD7 indicates duplex-PCR containing porcine and cattle DNA at a concentration of 0,0001 ng/μL; LN indicates a negative control. 169 bp is the product size for porcine and 227 is the product size for cattle species in this study.

confirmed that the detection at this DNA concentration is beyond sufficient to reveal species identity within processed meat products. The LOD in this study using *ND2* gene was markedly lower than previously investigated by Luo et al. (2008) in cattle, sheep, chicken, and pig at 0.1–0.2 ng, and duplex PCR to identify beef and buffalo by Gupta et al. (2012) at 0.001 ng.

3.4. Sequence details

Sequence analysis of PCR products was performed using BioEdit

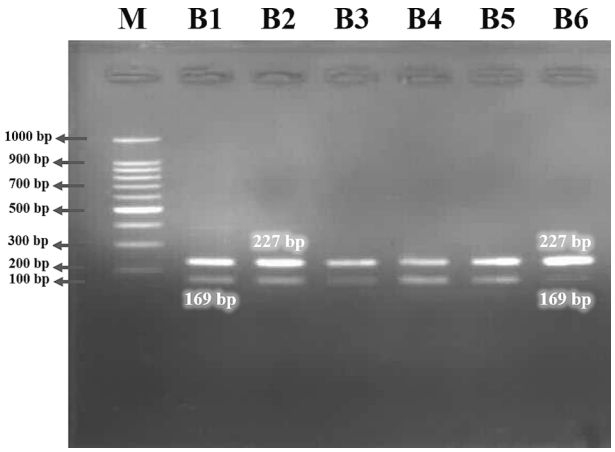

Fig. 5. Gel document visualization for validation of species detection in processed meat products targeting porcine and cattle mtDNA *ND2 gene*. M indicates 100 bp marker ladder; B1-B6 represent duplex-PCR targeting mtDNA *ND2* gene from various commercial meatball products sold in Surakarta, Indonesia. 169 bp is the product size for porcine and 227 is the product size for cattle species in this study.

7.2.6.1. The results of sequencing the ND2 primer after alignment analysis of porcine and beef samples are shown in S2. The alignment results in S2 revealed that the primer successfully restricted the target DNA sequence to be amplified. The porcine and cattle samples consisted of 168 and 227 bp, respectively. The reverse primer for porcine did not unintentionally attach to the cattle sequences, and vice versa, after confirming the PCR product sequence using the BLAST program. The BLAST program was used to investigate organisms with identical sequences by comparing input sequence data to the GenBank database (Ye et al., 2012). It was then confirmed that the sequences obtained in this study were identical to those used for designing the primers, as shown in S1. Pig sequences used in the present study were similar to those of Vietnamese pig breeds (KX982660.1) and (KX982658.1) in the mitochondrial section, with a degree of similarity of 98%. The cattle sequences used were similar to those of Bos Javanicus (JN632606.1) and Bos Gaurus (JN632604.1), with degrees of similarity of 97% and 93%, respectively. According to Diss (2003), these findings were considered significant because the primers successfully restricted the intended fragments to be amplified, not undesirable regions.

In addition, based on the sequencing results, both the universal forward primer and each reverse primer from porcine and beef were successfully attached to the intended sequence from the initial nucleotide limit point to the end of the nucleotide point to be amplified. Furthermore, it was also revealed that there was no mismatch either at the 3′- or 5′-end, as mismatching at both the 3′- and 5′-ends would be a critical mistake, causing an unspecific attachment and even amplification failure of the DNA fragment (Butsin *et al.*, 2020). The mismatch between the primers designed in the present study was <15% and was characterized as normal. The high rate of mismatching influences the melting temperature; therefore, a percentage of more than 15% would decrease the Tm by >15 °C (Matsunaga et al., 1999).

3.5. Commercial sample assessment of processed meat products

Primer specificity was examined against commercial beef meatballs labeled B1, B2, B3, B4, B5, and B6, which were randomly purchased from several traditional markets in Surakarta, Indonesia, and subjected to PCR. As shown in Fig. 5, all the examined samples were beef meatballs detected at 227 bp, confirming their origin as beef-derived products. However, as shown in Fig. 3, the gel documentation explicitly shows the existence of porcine DNA fragments detected at 168 bp with various intensities of bands consistent with the concentration of the mixture. This implies the improbability of meat producers' labels. It is widely understood that producers expect to achieve as much profit as possible by substituting higher-priced commercial meat with lower-priced meat (Wibowo et al., 2023). In addition, this result proved that the primer pairs designed in the present study were reliable and specifically amplified the intended target DNA fragment of *ND2*. A critical parameter is that the primer must only limit the target fragment, with no other amplification in the unintended region (Matsunaga et al., 1999). This also indicated that *ND2* can be a reliable genetic marker for species identification in meat and meat products.

4. Conclusion

Authenticating animal species within meat and meat products has never utilized duplex-PCR with *ND2 gene* as a marker. This study revealed that the assay exhibited a high sensitivity and specificity, having limit of detection at 0.0001 ng/μL. Gel documentation depicted the product size for pork and beef at 168 at 227 bp, confirming the result from *In-silico* experiment. The validated duplex-PCR technique utilizing the *ND2 gene* for the detection of bovine and porcine in processed meat products represents the fulfilment of assay for actual application. Successfully identifying mislabelling in wide range of commercial meatballs, the result of this study may bring practical benefits for policy maker, customer, and related stakeholders in the framework of fair-trade enforcement.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2023.100181.

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