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Multiplex PCR assay for species identification of meat and dairy products from buffalo (*Bubalus bubalis*), cattle (*Bos indicus* and *Bos taurus*), goat (*Capra hircus*), and sheep (*Ovis aries*)

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Abstract

Cases of fraudulent meat and dairy products have increased worldwide, especially in developing countries. To determine the misrepresented animal species, appropriate tools in routine monitoring should be available for food inspections. In the present work, a multiplex polymerase chain reaction assay for species identification of products from ruminants including buffalo, cattle, goat, and sheep was developed. The primer set KUMUT_cFarmSp1 was composed of five species-specific primers and a pair of positive-control primers. The primer set amplified 106-, 163-, 232-, and 308-bp specific fragments from the *cytochrome b* (*cyt b*) gene of buffalo, cattle, goat, and sheep, respectively, and 370-bp positive-control fragment from 16S ribosomal RNA (16S rRNA). The detection limit of this PCR assay is 0.1 ng of DNA template. The developed primer set exhibited strong specificity, sensitivity, robustness, and simplicity for food verification, thus indicating its usefulness for species verification in food quality control and law enforcement.

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Introduction

The most important domesticated animals in livestock agriculture are in the family Bovidae, specifically buffalo, cattle, goat, and sheep (Craine *et al.,* 2017). Several million tons of the meat and dairy products from these species are supplied to global markets, and employed as important food ingredients for many consumers worldwide (FAOSTAT, 2017). While the demand and market value of the meat and dairy products from ruminants are continuously increasing (Sans and Combris, 2015; IFCN, 2018), the incidents of food fraud have skyrocketed (Ballin, 2010; Handford *et al.,* 2016). The majority of food fraud reports regarding these ruminants' products have involved substitutions of products from less expensive species with / in those of more expensive species (Romero *et al.,* 1996; Song *et al.,* 2011; Abdul-Hassan and Tauma, 2014; Pinto *et al.,* 2015; 2017; Chuah *et al.,* 2016; Esteki *et al.,* 2019). Fraudulent meat and milk products represent global threats in many aspects such as public health, fair trade, law enforcement, as well as in religious, cultural, and ethnic considerations (Spink and Moyer, 2011; Alamprese *et al.,* 2013; Curll, 2015; Kamruzzaman, 2016; Cartin-Rojas, 2017).

To detect the cases of fraud in the meat and dairy industry, it is necessary to develop an effective investigation tool for species identification of raw materials in food products. Previously, a number of species-specific analytical techniques have been reported; these techniques employ four major types of analytical techniques namely chromatography, immunology, spectroscopy, and electrophoresis (Bargen *et al.,* 2014; Rodriguez *et al.,* 2010; Zelenakova *et al.,* 2010; Zhou *et al.,* 2016). Although all these techniques are reliable for species identification, their advantages and disadvantages vary depending on the user's objectives and applications, with the disadvantages of these methods are preventing them from being used routinely (Esteki *et al.,* 2019). In brief, protein-based techniques such as immunological and electrophoretic methods are less sensitive to heat-treated meat and dairy products due to the protein denaturation and high complexity of milk proteins. Additionally, these procedures are generally time-consuming, labour-intensive, and expensive (Murugaiah *et al.,* 2009; De *et al.,* 2011; Poonia *et al.,* 2016). Similarly, DNA-based techniques for species identification of food products such as PCR-RFLP, real-time PCR, DNA sequencing, and next-generation sequencing cannot be applied routinely simply because they are labour-intensive,

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time-consuming, and have a high cost per sample due to the requirements of expensive reagents and equipment (Kumar *et al.,* 2015; Reuter *et al.,* 2015; Rahmati *et al.,* 2016).

Recently, fraudulent meat and milk products cases have increased significantly, especially in the developing and underdeveloped countries (Azad and Ahmed, 2016; Tibola *et al.,* 2018). Therefore, appropriate monitoring tools that can be easily expedited and overcome the challenges faced by previously utilised techniques should be developed to detect fraudulent meat and dairy products. In the present work, a new primer set for multiplex end-point PCR and optimal PCR conditions were developed and validated for both meat and milk products. The target gene employed for the identification of each target species was *cytochrome b* (*cyt b*), which was previously applied to identify animal species in various taxa. Multiplex end-point PCR was designed for simultaneous amplification of species-specific fragments which may be detected by a simple DNA sizing method with agarose gel electrophoresis. Therefore, this DNA-based marker may provide a more robust species identification tool that can resolve future cases of fraudulent food, thus benefiting public health and fair trade worldwide.

Materials and methods

Samples

Three different groups of samples were collected between October 2018 and May 2019. The first group included blood samples obtained from buffalo (*Bubalus bubalis*), cattle (*Bos indicus*), goat (*Capra hircus*), and sheep (*Ovis aries*). These samples were used as DNA sources for primer testing, optimisation of PCR conditions, and positive controls. The second group contained blood samples of eight animal species including the four target species in group 1 along with pig (*Sus scrofa domesticus*), dog (*Canis lupus familiaris*), cat (*Felis catus*), and horse (*Equus ferus caballus*). Meat samples of six animal species were also obtained and analysed namely chicken (*Gallus gallus domesticus*), duck (*Anas platyrhynchos domestica*), goose (*Anser cygnoides domesticus*), East Asian bullfrog (*Hoplobatrachus rugulosus*), Nile tilapia fish (*Oreochromis niloticus*), and blue swimming crab (*Portunus pelagicus*). These samples were utilised to extract DNA for the primer set specificity test. The third group consisted of food product samples, as described in Table 1. All blood samples were collected from the Faculty of Veterinary Medicine, Mahanakorn University of Technology, Bangkok, Thailand, while meat and food product samples were purchased from local open markets and supermarkets in Bangkok, Thailand.

DNA extraction

The total genomic DNA extraction from blood and meat samples was performed using the FavorPrep™ Blood Genomic DNA Extraction Mini Kit and the FavorPrep™ Tissue Genomic DNA Extraction Mini Kit, respectively, following the manufacturers' protocols (Favorgen Biotech Corp., Taiwan).

DNA extraction from variety of meat and milk products was performed using a modified procedure that combined the Tris-EDTA method and salting-out method (Yalcinkaya *et al.,* 2017). All extractions from meat in processed foods were performed in 1.5-mL microcentrifuge tubes. Meat samples (30 - 50 mg) were added to 400 µL of

Table 1. Descriptions of labelled (expected) and detected species of commercial meat and dairy products.

No.	Meat product	Labelled/expected species	Detected species	No.	Dairy product	Labelled/expected species	Detected species
1.	Stir-fried buffalo and basil	buffalo	buffalo	15.	Raw buffalo milk	buffalo	buffalo
2.	Dry buffalo leather	buffalo	buffalo	16.	Raw cow milk	cattle	cattle
3.	Dry cow leather	cattle*	cattle	17.	Raw goat milk	goat	goat
4.	Roast beef	cattle	cattle	18.	Cow milk pasteurized	cattle	cattle
5.	Pastrami beef	cattle	cattle	19.	Goat milk UHT	goat	goat
6.	Meatball	cattle	cattle	20.	Cow milk powder	cattle	cattle
7.	Beef masaman curry	cattle	cattle	21.	Goat milk powder	goat	goat
8.	Beef biryani	cattle	cattle	22.	Cow-milk mozzarella	cattle	cattle
9.	Herby goat stew	goat	goat	23.	Buffalo-milk mozzarella	buffalo	buffalo
10.	Fried goat with garlic	goat	goat	24.	Cheddar cheese	cattle	cattle
11.	Lamb moussaka	lamb	lamb	25.	Goat cheese	goat	goat
12.	Roast lamb	lamb	lamb	26.	Iberico semicurado	cattle/goat/sheep	cattle/goat/sheep
13.	Meat dog food (animal feed)	cattle	cattle	27.	Yoghurt	cattle	cattle
14.	Lamb dog food (animal feed)	lamb	lamb	28.	Butter	cattle	cattle

*Cattle for meat and milk productions are most commonly *Bos indicus* and *Bos taurus*, respectively.

extraction buffer [20 mM Tris-HCl and pH 8.0; 20 mM EDTA and pH 8.0; 2% (w/v) SDS; and 40 µL of 10 mg/mL proteinase K (Amresco, Solon, OH, USA)]. The mixture was vortexed, and incubated at 65°C for 3 h. Next, 200 µL of 5 M NaCl was added, and samples were incubated at 65°C for 2 min, followed by incubation on ice for 10 min. Following centrifugation at 15,000 *g* for 3 min, the supernatant was transferred to a new tube. Equal volumes of saturated phenol were added, and then the mixture was vortexed and centrifuged at 15,000 *g* for 3 min. The collected upper aqueous phase was subsequently purified using successive extractions with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). DNA was precipitated by the addition of an equal volume of isopropanol alcohol, subsequently mixed by inversion, and centrifuged at 15,000 *g* for 15 min. The pellet was washed with 70% (v/v) ethanol, air-dried, and resuspended in 100 µL TE buffer (10 mM Tris, 0.1 mM EDTA, and pH 8.0).

Most steps of the DNA extraction protocol for solid and soft-solid dairy products were the same as those employed for meat products, except for the sample weight, extraction buffer volume used, and volume of 5 M NaCl which were 1 g, 5 mL, and 1 mL, respectively. For liquid milk, 50 mL of milk was centrifuged at 15,000 **g** for 3 min to obtain the milk pellet, which was subsequently handled as a solid or soft-solid dairy product.

Primer design

A multiplex PCR primer set for species identification of buffalo, cattle, goat, and sheep was developed based on the *cyt b* and 16S rRNA genes. Reference nucleotide sequences of *cyt b* and 16S rRNA are available in GenBank including NC_006295 (*Bubalus bubalis*), NC_005971 (*Bos indicus*), NC_006853 (*Bos taurus*), NC_005044 (*Capra hircus*), and NC_001941 (*Ovis aries*). The nucleotide sequences were aligned using Clustal W2 software (Larkin *et al.,* 2007). The primers were designed using the Primer-BLAST program (Ye *et al.,* 2012). The designed primers were synthesised by Macrogen Incorporation, Seoul, South Korea.

Optimisation of multiplex PCR

The optimisation of multiplex PCR conditions for the designed primer set was undertaken in two main steps. In the first step, five primer combinations (four species-specific combinations and a positive-control combinations) were tested independently by monoplex PCR to identify a single suitable annealing temperature for all combinations. In the second step, various multiplex PCR components which were determined according to the instructions of the Taq DNA polymerase manufacturer (Thermo Fisher Scientific, Lithuania, EU) were tested to increase their sensitivity while maintaining the specificity of the primer set.

The sizes of PCR products were determined using gel electrophoresis analysis on 2.5% (w/v) agarose for 70 min at 100 V in TBE buffer. The GeneRuler^{TM} Low Range DNA Ladder of 25 - 700 bp (Thermo Fisher Scientific, Lithuania, EU) was employed for size estimation of PCR products. DNA bands on the gel were visualised by staining with ethidium bromide (0.5 µg/mL). Gel images were recorded by a UV transilluminator using a BioDoc-ItTM Imaging System (UVP, Upland, California, USA).

Utility evaluation of the species-specific multiplex PCR

The specificity of the novel primer set and its PCR conditions were evaluated on the DNA template of four species for target ruminants (buffalo, cattle, goat, and sheep), four species of other mammals (pig, dog, cat, and horse), three species of domestic avians (chicken, duck, and goose), and three species of other food animals (frog, fish, and crab).

To determine the DNA template concentration limit, the sensitivity of the multiplex PCR was tested using serial dilutions of DNA templates (10, 1, 0.1, and 0.01 ng/µL). The sensitivity tests were performed using both single and mixed DNA templates of target species.

The verification of the new primer set for species identification of commercial meat and milk products was performed on 28 samples, including 14 samples of each meat and milk product (Table 1). Intact or diluted (if necessary) DNA solutions with concentrations in the range of 1 - 100 ng/µL were employed for the multiplex PCR.

Results

Primer design

Independent alignment of full-length *cyt b* and 16S rRNA genes from buffalo, cattle, goat, and sheep yielded lengths of 1,140 for *cyt b* and 1,584 bp for 16S rRNA. A multiplex PCR primer set for identifying target ruminant species that consisted of species-specific and positive-control primers was successfully developed based on the obtained alignment results. Oligonucleotide primers for species-specific amplification consisted of four species-specific forward primers of buffalo, cattle,

Table 2. Primer description, optimal concentration, and size of PCR products of the MUTKU_cFarmSp1 primer set.

No.	Primer name	Sequence (5'-3')	Target gene	Description	Optimal final concentration (µM)	Size (bp)
1.	BbCB_spH3	CATACATCCAAACAACGAAGTATG	*cyt b*	Buffalo specific forward primer	5.000	106
2.	BosCB_spH2	TAGGAGGAGTACTAGCCCTAGCCT	*cyt b*	Cattle specific forward primer	0.250	163
3.	ChCB_spH1	CCTCACATTAAACCTGAGTGGTAT	*cyt b*	Goat specific forward primer	1.400	232
4.	OaCB_spH4	CATGCTACTAGTACTATTCACGCCT	*cyt b*	Sheep specific forward primer	20.000	308
5.	Rumn_uniL1	CTGGYTGKCCTCCAATYCATG	*cyt b*	Common reverse primer	2.000	
6.	uFarm_16sH11	AAGACGAGAAGACCCTATGGAGCT	16S rRNA	Positive control forward primer	1.000	370
7.	uFarm_16sL12	GCTCCGGTCTGAACTCAGATCACG	16S rRNA	Positive control reverse primer	1.000	

goat, and sheep species and a common reverse primer, all of which were designed to target the *cyt b* gene (Figure 1a). Additionally, a pair of positive control primers specific to the conserved regions of the 16S rRNA gene were also designed (Figure 1b). The new primer set was named MUTKU_cFarmSp1 (Table 2)

Amplification of Multiplex PCR

The optimised PCR conditions employed with the MUTKU_cFarmSp1 primer set were of 20-µL total reaction volume containing 2 µL 10X Taq Buffer, 1.25 mM $MgCl_2$, 0.2 mM of each dNTP, 2 units Taq DNA polymerase (Thermo Fisher Scientific, Lithuania, EU), 0.250 - 20.000 µM of each primer (Table 2), and 1 µL of each DNA sample for both single and multiple template PCRs. The thermal cycler parameters were: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, elongation at 72°C for 30 s, and final elongation at 72°C for 30 s. This PCR assay amplified 106-, 163-, 232-, and 308-bp specific fragments for buffalo, cattle, goat, and sheep, respectively (Figure 1). At the same time, the 370-bp positive-control fragment could be amplified alongside all species-specific fragments in the same PCR tube.

Specificity

Verification of the MUTKU_cFarmSp1 primer set was undertaken to assess the possibility of species misidentification. PCR amplification using DNA templates from the 14 animal species mentioned earlier showed simultaneous amplification of both species-specific and positive-control fragments of buffalo, cattle, goat, and sheep DNA templates. Only positive-control fragments were amplified when the template DNA consisted of pig, dog, cat, horse, goose, bullfrog, or Nile tilapia fish total DNA. However, the templates DNA from chicken, duck, and blue swimming crab yielded no amplification. Species-specific bands and positive-control bands could not be detected (Figure 2). Unexpected fragments or misidentified fragments were also not detected in any PCR products.

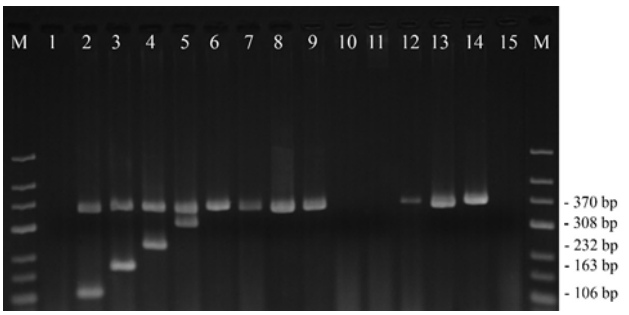

Figure 2. Agarose gel electrophoresis of multiplex PCR using the MUTKU_cFarmSp1 primer set. Lane M = Low Range GeneRuler™, 1 = negative control, 2 = buffalo, 3 = cattle, 4 = goat, 5 = sheep, 6 = pig, 7 = dog, 8 = cat, 9 = horse, 10 = chicken, 11 = duck, 12 = goose, 13 = East Asian bullfrog, 14 = Nile tilapia fish, and 15 = blue swimming crab.

Sensitivity

The sensitivity of the MUTKU_cFarmSp1 primer set under its optimal PCR conditions was verified on serial dilutions of single and mixed DNA templates (Figure 3). The species-specific and control fragments could be detected by single-template multiplex PCR using 0.01 ng/µL of buffalo, cattle, and goat DNA samples, whereas 0.1 ng/µL was required for the sheep DNA sample. Therefore, the 0.1 ng/µL concentration of template DNA is considered to be the minimum DNA concentration that can be analysed by this primer set.

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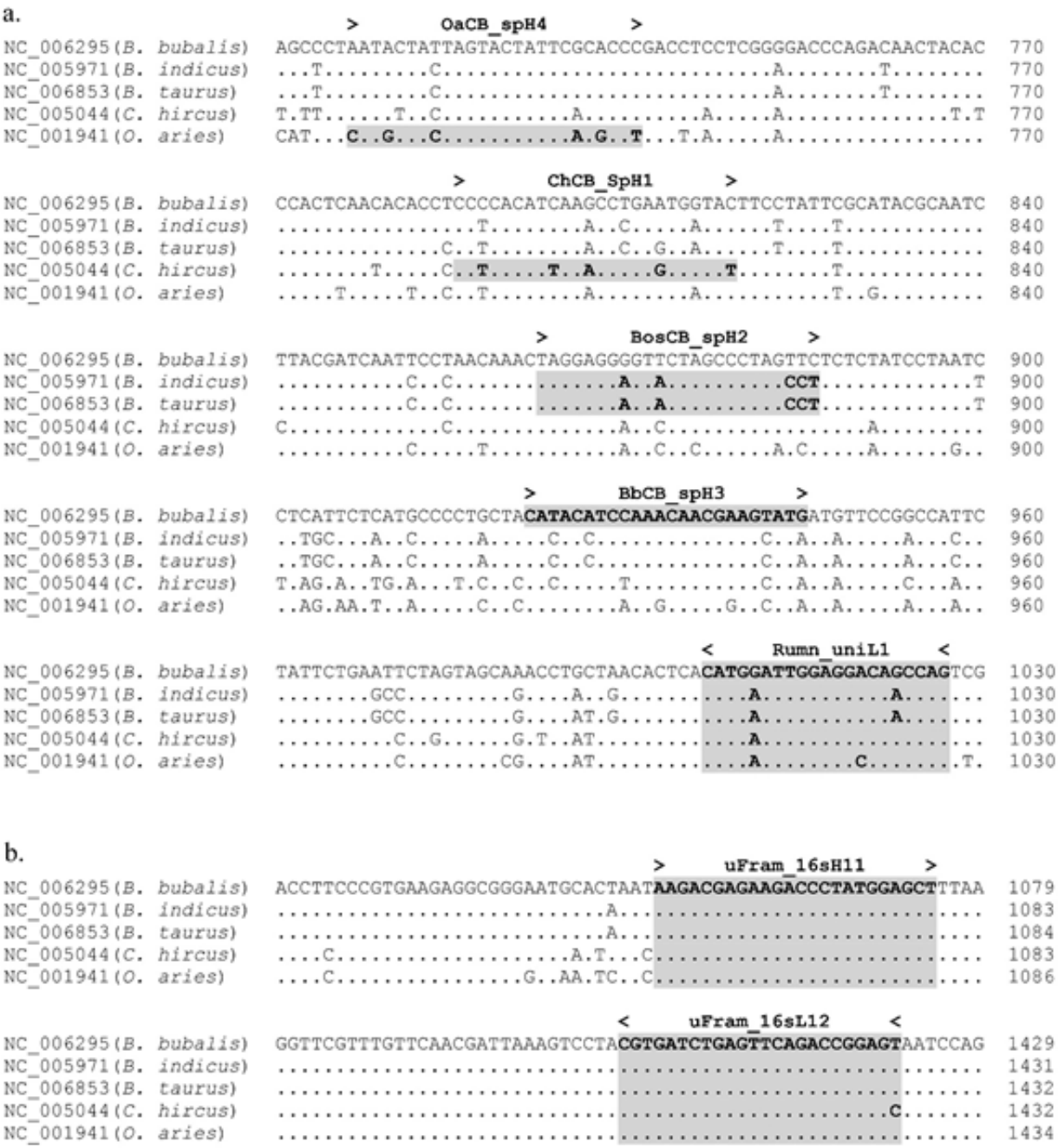

Figure 1. Alignment of *cyt b* (a) and 16S rRNA (b) sequences from *Bubalus bubalis, Bos indicus, Bos taurus, Capra hircus*, and *Ovis aries*. Identical bases to the top sequence are shown by dots. The binding site of each primer is shown by the shaded area. Primer names and their direction are shown by symbols "<" and ">".

Assessment of commercial meat and dairy products

The primer set was tested on several types of commercial meat and milk food products. Gel images (Figure 4) showed specific fragments of meat or milk species in all food samples, while a positive control band (370 bp) could be detected in all food samples, except for dog meat. Fraudulent food was not detected in the food samples tested in the present work.

Discussion

In the present work, a multiplex PCR assay using the primer set MUTKU_cFarmSp1 (Table 2) was investigated as a species-specific DNA-based assay for the detection of any traces of farmed ruminants including buffalo, cattle, goat, and sheep in commercial food products (Figures 2 and 4); this assay can be used instead of a number of existing protein-based or DNA-based assays. The major disadvantages of the previous methods are expensive, time-consuming, labour-intensive, and produce results that are difficult to interpret. These drawbacks are the major obstacles facing the routine application of MUTKU_cFarmSp1 in basic laboratories of many

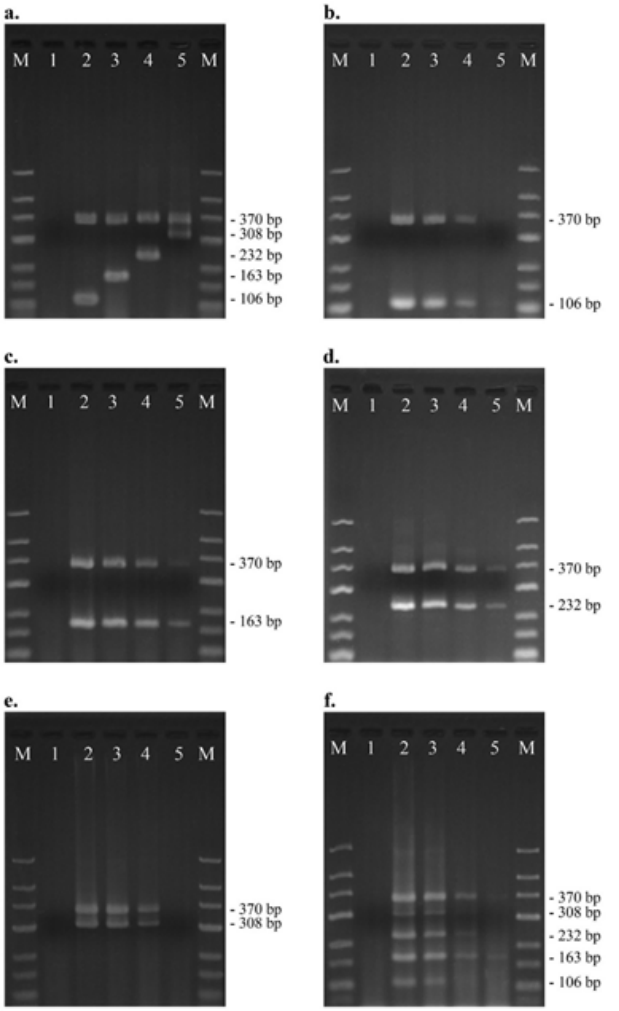

Figure 3. Sensitivity test of multiplex PCR using the MUTKU_cFarmSp1 primer set on buffalo (b), cattle (c), goat (d), sheep (e), and a mixture of DNA templates of four target species (f). Lanes 2 - 5 of (b) - (f) show serial dilutions of DNA template in PCRs at 10, 1, 0.1, and 0.01 ng, respectively. Lane M and 1 of all gel images are Low Range GeneRuler™ and negative control, respectively. (a) shows size comparisons of species-specific and positive-control bands of all target species.

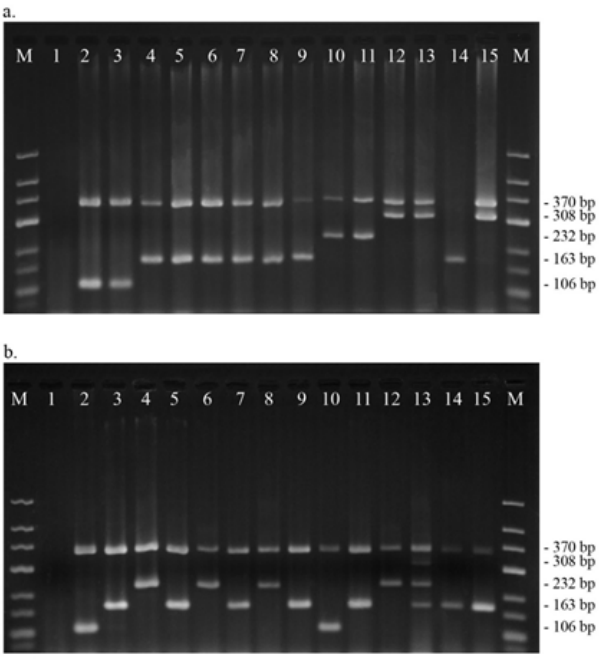

Figure 4. Species identification of meat and dairy products by the multiplex PCR assay using MUTKU_cFarmSp1. Meat product assays (a) in lane 2 = stir-fried buffalo and basil, 3 = dry buffalo leather, 4 = dry cow leather, 5 = roast beef, 6 = pastrami beef, 7 = meatball, 8 = beef masaman curry, 9 = beef biryani, 10 = herby goat stew, 11 = fried goat with garlic, 12 = lamb moussaka, 13 = roast lamb, 14 = meat dog food (animal feed), and 15 = lamb dog food (animal feed). Dairy product assays (b) in lane 2 = raw buffalo milk, 3 = raw cow milk, 4 = raw goat milk, 5 = cow milk pasteurised, 6 = goat milk UHT, 7 = cow milk powder, 8 = goat milk powder, 9 = cow-milk mozzarella, 10 = buffalo-milk mozzarella, 11 = cheddar cheese, 12 = goat cheese, 13 = iberico semicurado, 14 = yogurt, and 15 = butter. Lane M and 1 of (a) and (b) are Low Range GeneRuler™ and negative control, respectively.

developing countries where fraudulent meat and dairy products are often found (Handford *et al.,* 2016). Therefore, MUTKU_cFarmSp1 was intentionally developed as a DNA marker that can be routinely employed for species detection with high specificity, sensitivity, robustness, and more importantly, simplicity. These properties are key characteristics that may enable MUTKU_cFarmSP1 to become a powerful detection instrument against cases of fraudulent food for the studied ruminants (buffalo, cattle, goat, and sheep) because this assay features a potent detection tool and accurate data for food components even when they are present only in trace amounts, and it can also identify down to the species level. The information obtained by this method may be a useful complement to the blockchain technology currently being employed in the food supply chain (Galvez *et al.,* 2018).

The sensitivity of the species-specific simplex and multiplex PCRs has typically been reported as a minimum requirement of DNA template per reaction for successful amplification of target DNA fragments. Recently, the sensitivity of species-specific PCRs has been reported through two different parameters based on different detection methods. For the first parameter, total genomic DNA, including both nuclear and organelle DNA in the PCR, is used. The range of detection sensitivity based on the amount of total genomic DNA was reported to be 5 - 0.001 ng (Zha *et al.,* 2010; Dai *et al.,* 2015), which included 0.1 ng sensitivity of MUTKU_cFarmSP1 (Figure 3). For the second parameter, the mtDNA copy number is utilised to set a sensitivity limit (Tobe and Linacre, 2008). For a direct-multiplex PCR assay

for meat identification, the sensitivity limit was reported to be 12,500 mitochondrial copies, which was equivalent to 7 fg (Kitpipit *et al.,* 2014). To obtain the mtDNA copy number, a complex direct evaluation of total non-human mammalian mtDNA must be performed. However, it has been commonly recognised that sensitivity limits reported by both parameters provide good references of minimal DNA amount required for PCR-based detection methods; as a result, the total genomic DNA remains the preferred parameter for sensitivity limit due to its simplicity of detection.

According to the robustness testing of the MUTKU_cFarmSp1 primer set on real samples, the primer set was able to identify its target species from various types of commercial meat and milk products (Table 1). Typically, the robustness of species-specific multiplex PCR for food product identification is primarily determined by the lengths of PCR products. The optimal lengths of the target fragments in the present work ranged from 100 to 400 bp (Table 2). Long PCR products (> 500 bp) would be adversely affected by DNA fragmentation, which can be caused by both food processing steps or particular ingredients in the food (Pereira *et al.,* 2008; Piskata *et al.,* 2017); while short products (< 100 bp) are difficult to analyse by agarose gel electrophoresis but can be easily managed using polyacrylamide gel electrophoresis (Pereira *et al.,* 2008; Barril and Nates, 2012). Moreover, the MUTKU_cFarmSp1 primer set not only comprises species-specific primers but also includes a pair of positive control primers, which can notably simplify the interpretation of the results by providing clear verification of the presence of all PCR components, the absence of PCR inhibitors, and normal operation of the thermal cycler. Furthermore, the position of the positive control PCR products on a gel image could be used as a secondary size reference of species-specific bands in addition to the primary size reference provided by the commercial DNA ladders (Figure 2 and 4). Despite the advantages of having the positive control primers in species identification using multiplex primer sets, there have been only a small number of reports that included them in the identification of food products (Catanese *et al.,* 2010; Hellberg *et al.,* 2010; Sangthong *et al.,* 2014; Suwannarat *et al.,* 2017), and in some meat and dairy products from farmed ruminants (Xue *et al.,* 2017).

Finally, there are two considerations to be contemplated when using the MUTKU_cFarmSp1 primer set. First, the time requirement for species detection is approximately 8 h, including 4 h for DNA extraction, 2 h for PCR, and 2 h for gel electrophoresis. This time period is longer than those of several previously reported methods, such as real-time PCR (Sawyer *et al.,* 2003; Sakalar and Kaynak, 2016), the Lab-on-a-Chip electrophoretic system (Ali *et al.,* 2015), and the direct multiplex PCR assay (Kitpipit *et al.,* 2014; Thanakiatkrai *et al.,* 2019). Although a rapid protocol is undoubtedly an advantage, if one takes into account that the main application of this primer set provides a simple and reliable method for accurate species identification that might be used for law enforcement and for quality control of meat and dairy products, then a small delay of a few hours is insignificant, as the time frame of inspection would still be within day(s) or week(s), similar to the old methods (FAO, 1993). Second, the MUTKU_cFarmSp1 primer set can easily and economically be upgraded to become high throughput by utilising an automated separation system of capillary electrophoresis. For this upgrading, only two primers (first: Rumn_uniL1 and second: uFarm_16sH11 or uFarm_16sL12) need to be labelled by fluorescent dyes, such as 6-carboxyfluorescein (6-FAM). The automated separation for the species identification of food products is suitable for large-scale service laboratories.

Conclusion

In the present work, a multiplex PCR primer set named MUTKU_cFarmSp1, and its optimised PCR conditions for species identification of buffalo, cattle, goat, and sheep were developed. This assay was designed to be a simple protocol with high specificity, high sensitivity, and strong robustness in the detection of target species. The primer set can be effectively tested not only on various meat and dairy products, but also on animal feed and leather products. This assay can be a useful application in routine food surveillance performed by both the private companies and government agencies.

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