Development of RAPD-PCR assay for identifying Holstein, Angus, and Taiwan Yellow Cattle for meat adulteration detection

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Abstract Incidents of food fraud have occurred worldwide, particularly in the form of meat adulteration. In this study, molecular probes were developed using the Random amplification of polymorphic DNA (RAPD) polymerase chain reaction (PCR) technique in order to identify three beef subspecies—Holstein, Angus, and Taiwan Yellow Cattle. Four RAPD-PCR 10-nucleotide primers were chosen out of a total of 60 primers. The selection was based on the reproducibility of species-specific amplicons able to detect various origins of cattle breeds. The results demonstrated that primer OPK12 produced three unique amplicons (1100 bp, 1000 bp and 480 bp) in Holstein; primer OPK14 generated one amplicon that only appeared in Holstein and Angus (200 bp); primer OPK19 amplified two species-specific amplicons in Holstein measuring 550 bp and 650 bp, respectively. However, due to the relatively lower repeatability of RAPD-PCR, higher and more specific testing repeats were required to increase the accuracy of the conclusion.

Keywords RAPD-PCR · Food adulteration · Cattle · Meat product · Species identification

Introduction

Beef has become one of the most popular products in the food industry. However, food fraud of late has grown to become a severe issue in many regions, including Taiwan (Peng et al., 2017). Mixing and/or replacing high-value species with low-value ones for economic gain has become a prominent form of food alteration in the meat industry (Fajardo et al., 2008; Soares et al., 2013), and the issue has gradually become a potential crisis in the Taiwanese beef market. As a result, developing an effective probe is necessary to guarantee the purity of meat products, protect human health, and eradicate economic fraud. In short, species identification is of great importance in terms of conducting food tracking and ensuring food safety, and its practical applicability in the meat industry is obvious. This trend is reflected in the numerous, diverse, and cutting-edge approaches for species tracking that have been recently published (Kim and Kim, 2018; Kitpipit et al., 2014; Kumar et al., 2015; Rahmati et al., 2016).

Species tracking using protein and DNA testing have been applied widely in various food industries, such as coffee (Combes et al., 2018), minced beef products (Ropodi et al., 2017; Velioglu et al., 2018) and honey (Sobrino-Gregorio et al., 2018). In addition, many researches have done on identification of meat species in different types of meat products (Kim and Kim, 2017; Thanakiatkrai et al., 2019). However, distinguishing species through protein analysis may sometimes lack in reliability because of heating and chemical reactions, which result in the denaturation of proteins in meat products

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(Rastogi et al., 2007). Nonetheless, using polymerase chain reaction (PCR) has a number of advantages, such as its relatively high accuracy and sensitivity and the relative ease and simplicity of sample preparation (Chen et al., 2014; Rahman et al., 2015). Among the various PCR approaches, Random Amplification of Polymorphic DNA (RAPD) is regarded as one of the faster and cheaper techniques to identify meat components, and several applications have been previously reported via this assay (Kaur et al., 2017; Keeratipibul and Techaruwichit, 2012; Kwon et al., 2009; Martinez and Yman, 1998). Unlike other PCR techniques, such as Real-time PCR (Xu et al., 2018) and PCR-RFLP (Abdel-Rahman and Ahmed, 2007), no knowledge of the DNA sequence of the targeted genome is required as the primers will randomly bind on the genomic sequence. As a result, RAPD provides an excellent opportunity to investigate and compare the DNA of biological species that have been ignored by previous methods.

In this study, specific primers were selected as probes for detecting three different subspecies of beef meat through RAPD, and target primers were picked out of sixty primers. Furthermore, the objective of this study is to develop an effective, simple, and low-expenditure assay to identify three different types of beef commonly available in the Taiwan beef market—Holstein, Angus, and Taiwan Yellow Cattle.

Materials and methods

Preparation of meat samples

Meat samples were procured from chain supermarkets and retailers from cities located in central and southern Taiwan. Fifteen raw meat samples of *Bos Taurus* (Angus) were purchased directly from chain supermarkets in the cities of Kaohsiung and Taichung. Fifteen samples each of *Bos Taurus* (Holstein) and *Taiwan Zebu* (Taiwan Yellow Cattle) were obtained from local retailers in Changhua and Chiayi, respectively. All 45 samples were verified with the certificate of Taiwan traceability agricultural product for the purpose of ensuring authenticity. The meat samples

Table 1 The primers used for random amplified polymorphic DNA (RAPD) PCR

Primers	Nucleotides (5' → 3')
OPK12	5'TGGCCCTCAC3'
OPK14	5'CCCGCTACAC3'
OPK19	5'CACAGGCGGA3'

were immediately stored at -20°C after procurement to await DNA extraction.

DNA extraction

DNA was extracted from the samples using the DNeasy Blood and Tissue Kit (Qiagen, Germany). The procedure for procuring DNA from the samples was based on the manufacturer's manual. Briefly, before extraction, each sample (25 mg) was cut into small pieces and put into a 1.7 mL microcentrifuge tube. Twenty μL proteinase K working solution and 180 μL Buffer ATL were added per sample and mixed using vortexing. The samples were incubated at 56°C overnight, followed by the addition of 200 μL Buffer AL and 200 μL ethanol (99%). The solution was then thoroughly mixed by vortexing. Five hundred μL of mixture was pipetted and transferred into the DNeasy Mini spin column placed in a 2 mL collection tube. The mixture was then centrifuged at 10,000 rpm for 1 min. After the flow-through mixture was discarded, 500 μL Buffer AW1 was added and centrifuged for 1 min at 10,000 rpm. After discarding the spin down mixture, Buffer AW2 was added in a volume of 500 μL and centrifuged for 4 min at 12,500 rpm. Eventually, DNeasy Mini spin column was transferred and placed in a clean 1.7 mL microcentrifuge tube, followed by 50 μL Buffer AE directly added. The tube was then centrifuged for 1 min at 8000 rpm, following the incubation at room temperature for 10 min. The DNA concentration and purity were measured via a NanoDropTM 1000 spectrophotometer (Thermo Fisher Scientific Inc. Waltham, MA, USA) after extraction. The samples were finally stored at -20°C to await testing.

Table 2 Oligonucleotide primers designed based on the Primer3 output designing tool

Primers	Nucleotides (5' → 3')	Products size (bp)
OPK12-F	5' CCCACTAGGCCATTTTGAGA 3'	672
OPK12-R	5' AGATTACGTGCCCAGTGTCC 3'	
OPK14-F	5' ACCCGTTTCATTGCTTTGTTC 3'	976
OPK14-R	5' TTGTGTAGGGCCTTCTCTGG 3'	
OPK19-F	5' TGGCCACCTGGAGTATAAGG 3'	612
OPK19-R	5' GGGGAGAGGTCAAACACAGA 3'	

Table 3 Comparison of assays' results in terms of the number of bands generated in sixty primers

Primer code	No. of bands amplified in			No. of bands for	
	H	A	Y	Exclusive fragment	Shared fragment
OPI 01	0	0	0	0	0
OPI 02	8	8	0	0	8
OPI 03	7	7	0	0	7
OPI 04	0	0	0	0	0
OPI 05	0	0	0	0	0
OPI 06	1	0	0	1	0
OPI 07	0	0	0	0	0
OPI 08	0	0	0	0	0
OPI 09	3	3	0	0	3
OPI 10	0	0	1	1	0
OPI 11	0	0	0	0	0
OPI 12	9	9	8	0	9
OPI 13	0	0	0	0	0
OPI 14	0	0	0	0	0
OPI 15	0	0	0	0	0
OPI 16	0	0	0	0	0
OPI 17	2	0	0	0	0
OPI 18	0	0	0	0	0
OPI 19	3	0	0	0	0
OPI 20	0	0	0	0	0
OPJ 01	0	0	0	0	0
OPJ 02	0	0	0	0	0
OPJ 03	0	0	0	0	0
OPJ 04	0	0	0	0	0
OPJ 05	0	1	0	1	0
OPJ 06	0	0	0	0	0
OPJ 07	0	0	0	0	0
OPJ 08	0	0	0	0	0
OPJ 09	1	1	0	0	1
OPJ 10	0	2	0	2	0
OPJ 11	0	0	0	0	0
OPJ 12	0	0	0	0	0
OPJ 13	0	0	0	0	0
OPJ 14	0	0	0	0	0
OPJ 15	0	1	0	1	0
OPJ 16	0	0	0	0	0
OPJ 17	0	0	0	0	0
OPJ 18	0	3	0	3	0
OPJ 19	5	5	0	0	5
OPJ 20	9	3	0	6	3
OPK 01	0	0	0	0	0
OPK 02	0	0	0	0	0
OPK 03	0	0	0	0	0
OPK 04	3	0	0	3	0
OPK 05	0	0	0	0	0
OPK 06	0	0	0	0	0
OPK 07	0	6	0	6	0
OPK 08	4	0	0	4	0

Table 3 continued

Primer code	No. of bands amplified in			No. of bands for	
	H	A	Y	Exclusive fragment	Shared fragment
OPK 09	0	0	0	0	0
OPK 10	0	0	0	0	0
OPK 11	0	0	0	0	0
OPK 12	0	6	0	6	0
OPK 13	0	0	0	0	0
OPK 14	0	7	0	7	0
OPK 15	0	0	0	0	0
OPK 16	0	0	0	0	0
OPK 17	0	0	0	0	0
OPK 18	0	0	0	0	0
OPK 19	0	2	0	2	0
OPK 20	0	0	0	0	0

H: Holstein, A: Angus, Y: Taiwan yellow cattle

Random amplified polymorphic DNA (RAPD) PCR

Primers OPK12, OPK14 and OPK19 obtained from Operon RAPD 10 Mer Kit (CosmoGenetech Inc. Seoul, South Korea) for RAPD-PCR are shown in Table 1. The DNA amplification was carried out in a total volume of 20 µl reagent, which contained the following reagents: 10 µl PCR Master Mix (2X) (Thermo Fisher Scientific Inc. Waltham, MA, USA), Nuclease-Free Water (Thermo Fisher Scientific Inc. Waltham, MA, USA), 200 ng DNA template and 1 µl of 5 µM Primer. The RAPD-PCR was processed on a Bio-Rad T100™ thermal cycler (Bio-Rad Laboratories, Inc. Hercules, CA, USA), and the cycling program was as follows: 94 °C for 5 min, followed by 45 cycles at 94 °C for 1 min, 36 °C for 1 min and 72 °C for 1 min with a final extension of 72 °C for 10 min. A scalpel was used for excising gel fragment, and the amplified product was extracted using the Gel/PCR DNA Fragments Kit (Geneaid Biotech Ltd. New Taipei City, Taiwan).

Nucleotide sequencing

To detect DNA polymorphisms, the exclusive amplicon shown on the electrophoresis was excised via the recommended protocol of the Gel/PCR DNA Fragments Kit (Geneaid Biotech Ltd. New Taipei City, Taiwan). Nucleotides sequencing was carried out with a Capillary Automatic DNA Sequencer (ABI 3730, Thermo Fisher Scientific Inc. Waltham, MA, USA).

Oligonucleotide primers design and amplification

After validating the nucleotide sequences obtained from the excised RAPD amplicon, oligonucleotide primers were

designed to target the exclusive amplicons using Primer3 tool (<http://frodo.wi.mit.edu>) and were synthesized by Genomics Biological Technology and Services Co., Ltd., New Taipei City, Taiwan. The sequences are shown in Table 2. The PCR amplification was performed on a Bio-Rad T100™ thermal cycler (Bio-Rad Laboratories, Inc. Hercules, CA, USA) using the following procedure: initial denaturing for 3 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 61 °C, 1 min at 72 °C, and 10 min at 72 °C (final extension).

Agarose gel electrophoresis

All amplified PCR amplicons were electrophoresed on an agarose gel. Agarose gel (1.5%) was manufactured in a microwave oven by dissolving appropriate quantities of agarose in 1 × TBE buffer. 6 µl of Safeview (GMbiolab Co, Ltd. Taipei, Taiwan) was subsequently added to molten agarose before pouring the gel into the tray. After electrophoresis, DNA amplicons in the agarose gel were visualized and photographed through a Gel Documentation Systems (Bio-Rad Laboratories, Inc. Hercules, CA, USA).

Results and discussion

Scanning of RAPD primers

Out of the 60 primers that resulted from RAPD screening, four primers (OPK07, OPK12, OPK14, OPK19) were selected based on multiple criteria that included the appearance of the unique bands among the three species and the display of apparent patterns. However, fragments appear in two or more species simultaneously and without significant pattern were not considered. The 60 primers

Fig. 1 **A** RAPD-PCR amplification of pattern showing distinction between sub-species. M: 100 bp plus DNA ladder, lane 1: primer OPK07 and Holstein DNA, lane 2: primer OPK07 and Angus DNA, showing a unique amplicon with the molecular weight of 1200 bp (shown in arrow mark), lane 3: primer OPK07 and Taiwan Yellow Cattle DNA. **B** RAPD-PCR amplification of pattern showing distinction between sub-species. M: 100 bp plus DNA ladder, lane 1: primer OPK12 and Holstein DNA, lane 2: primer OPK12 and Angus DNA, showing a unique amplicon with the molecular weight of 700 bp (shown in arrow mark), lane 3: primer OPK12 and Taiwan Yellow Cattle DNA, lane 4: primer OPK14 and Holstein DNA, lane 5: primer OPK14 and Angus DNA, showing a unique amplicon with the molecular weight of 1800 bp (shown in arrow mark), lane 6: primer OPK14 and Taiwan Yellow Cattle DNA, lane 7: primer OPK19 and Holstein DNA, lane 8: primer OPK19 and Angus DNA, showing a unique amplicon with the molecular weight of 600 bp (shown in arrow mark), lane 9: primer OPK19 and Taiwan Yellow Cattle DNA

tested for the three species generated a total of 128 RAPD fragments with 69 bands in OPI series, 31 bands in OPJ series and merely 28 bands in OPK series. “Specifically, primer OPI series generated 2 exclusive bands and 27 shared bands among three sub-species. OPJ series generated 13 exclusive bands and 9 shared bands. OPK series showed 28 exclusive bands and no shared bands. The

results are summarized in Table 3. Although many primers have produced exclusive fragments among the three species, most of them were unclear amplicons; therefore, these primers were eliminated from the selection. In Fig. 1A, primer OPK07 produced more amplification fragments in comparison with other primers (primer OPK12, OPK14, OPK19), with amplicons ranging from 800 to 2200 bp in

Fig. 2 Results of PCR fingerprinting by selective primer. **A** H: Holstein, A: Angus, Y: Taiwan Yellow Cattle. M: 100 bp Plus DNA ladder, lane 1: unique amplicons (1000 bp, 1100 bp and 480 bp) in Holstein by primer OPK12 (shown in arrow mark), lane 2 Angus with primer OPK12, lane 3: Taiwan Yellow Cattle with primer OPK12. **B** Amplicon showed only in Holstein and Angus. H: Holstein, A: Angus, Y: Taiwan Yellow Cattle. M: 100 bp Plus DNA ladder, lane 1: amplicon with 180 bp molecular weights in Holstein by primer OPK14 (shown in arrow mark), lane 2: amplicon with 180 bp molecular weights in Angus by primer OPK14 (shown in arrow mark), lane 3: Taiwan Yellow Cattle with primer OPK14. **C** H: Holstein, A: Angus, Y: Taiwan Yellow Cattle. M: 100 bp Plus DNA ladder, lane 1: unique amplicons (550 bp, 650 bp) in Holstein by primer OPK19 (shown in arrow mark), lane 2 Angus with primer OPK19, lane 3: Taiwan Yellow Cattle with primer OPK19

Angus only. In Fig. 1B, primer OPK12 displayed 6 fragments with molecular weights ranging between 700 and 2000 bp, while primer OPK19 displayed only two bands (600 bp, 250 bp). However, six amplicons were produced by primer OPK14, and they ranged from 700 to 1800 bp, with only one of these bands (1800 bp) showing clearly. All amplicons mentioned above that were produced in Angus exclusively. Several bands (1200 bp from OPK07, 700 bp from OPK12, 1800 bp from OPK14 and 600 bp from OPK19) shown in arrows were excised from the gel in order to obtain the sequence for specific primer design subsequently. All excised amplicons were exclusive and demonstrated notably visible bands among the three species.

RAPD marker development

Exclusive band patterns of PCR amplicon were observed in the three tested subspecies of cattle, which produced from the designed primers and traditional PCR cycling (Fig. 2). Primer OPK12 propagated three unique and noticeable amplicons with molecular weights of 1100 bp, 1000 bp and 480 bp in Holstein. Primer OPK14 produced one amplicon with 200 bp in Holstein and Angus; however, the band was not present in Taiwan Yellow Cattle. Primer OPK19 generated three manifest and exclusive amplicons (550 bp, 650 bp) in Holstein that were absent in Angus and Taiwan Yellow Cattle. Primer OPK07, however, failed to amplify any amplicons in the assay. In summary, the results of the experiment demonstrate that primers OPK12, OPK14, and OPK19 have the potential to serve as probes capable of distinguishing different sub-species in beef products.

Table 4 The frequency of the RAPD-PCR pattern occurrence after five consecutive assays

Primer no.	Species	Number of assays					Repeatability (%)
		First	Second	Third	Fourth	Fifth	
OPK12	Holstein	+	+	–	–	–	40
OPK12	Angus	–	–	–	–	+	20
OPK12	Taiwan Yellow Cattle	–	–	–	–	–	0
OPK14	Holstein	+	+	–	–	–	40
OPK14	Angus	–	–	–	–	–	0
OPK14	Taiwan Yellow Cattle	–	–	–	–	–	0
OPK19	Holstein	–	+	–	–	–	20
OPK19	Angus	–	–	–	–	–	0
OPK19	Taiwan Yellow Cattle	–	–	–	–	–	0

“+” Means the same fragment observed in the assay. “–” Means none of the fragment observed in the assay

False detection verification

False detection verification was conducted to verify the accuracy of the RAPD-PCR and the issue of cross-contamination. Raw meat samples of three species were mixed and cut gently in the tube for DNA extraction following the same process in the materials and methods section. The expected fragments for Primer OPK12, OPK14 and OPK19 reoccurred as that of the DNA extraction from an only single species (Figures shown in the “Appendix”). The verification procedures revealed that this technique is capable of avoiding false positive results, and can distinguish cattle sub-species in a mixed sample.

Advantages and disadvantages of RAPD-PCR

Due to the fact that RAPD does not require further DNA sequence of the target organism before the assays, it is cheap and convenient to develop markers compared to other molecular techniques. However, several drawbacks have been reported in previous studies. One drawback is that vital differences situated on non-amplified areas could be missed due to the length of oligonucleotide primers (10 bp) that constitute only a limited part of genome (Shehata, 2004). Another disadvantage is the fact that the fragments lack high reproducibility because of multiple factors, such as intra-polymorphism of species or cycling conditions (Koh et al., 1998). In addition, the reproducibility was not improved even with the adjustment of the annealing temperature (Perez et al., 1998). Our assays also confirmed the downside mentioned above, as Table 4 demonstrates. The recurrence rates for RAPD-PCR

amplicons are relatively low in primer OPK12 (40% for Holstein, 20% for Angus, and 0% for Taiwan Yellow Cattle, respectively). In addition, a similar phenomenon can be seen in primers OPK14 and OPK19. The repeatability for OPK14 Holstein is 40% and that for OPK19 is merely 20%. A 0% repetition rate is shown for Angus and Taiwan Yellow Cattle for both primer OPK14 and primer OPK19.

The potential for further application

Due to its high genome similarity, varieties characterization under the sub-species level is relatively more challenging. To our knowledge, this is the first study demonstrating that the RAPD-PCR assay is an effective and relatively simple method to trace the meat origin from three sub-species cattle (Holstein, Angus, and Taiwan Yellow Cattle). The technique can be easily implemented in a normal laboratory and can be used without knowing the DNA sequence of the species beforehand and without the use of high throughput equipment. Furthermore, the method possesses wider applications in terms of verifying other prevalent meat species and tracing adulteration in other commercial meat products. We believe that the technique presented in this study will not only provide a reliable strategy for improving beef sub-species identification, but also enhance the law enforcement for meat adulteration.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Appendix

See Figs. 3, 4, 5.

Fig. 3 Results of PCR fingerprinting of false detection. H: Holstein, A: Angus, Y: Taiwan Yellow Cattle, HAY: Holstein + Angus + Taiwan Yellow Cattle. M: 100 bp Plus DNA ladder, lane 1: unique amplicons (1000 bp, 1100 bp and 480 bp) in Holstein by primer OPK12 (shown in arrow mark), lane 2: Angus with primer OPK12, lane 3: Taiwan Yellow Cattle with primer OPK12

Fig. 4 Results of PCR fingerprinting regarding false detection. Amplicon showed only in Holstein and Angus. H: Holstein, A: Angus, Y: Taiwan Yellow Cattle, HAY: Holstein + Angus + Taiwan Yellow Cattle. M: 100 bp Plus DNA ladder, lane 1: amplicon with 180 bp molecular weights in Holstein by primer OPK14 (shown in arrow mark), lane 2: amplicon with 180 bp molecular weights in Angus by primer OPK14 (shown in arrow mark), lane 3: Taiwan Yellow Cattle with primer OPK14

Fig. 5 Results of PCR fingerprinting regarding false detection. H: Holstein, A: Angus, Y: Taiwan Yellow Cattle, HAY: Holstein + Angus + Taiwan Yellow Cattle. M: 100 bp Plus DNA ladder, lane 1: unique amplicons (550 bp, 650 bp) in Holstein by primer OPK19 (shown in arrow mark), lane 2: Angus with primer OPK19, lane 3: Taiwan Yellow Cattle with primer OPK19

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