Ann. Anim. Sci., Vol. 16, No. 2 (2016) 415–424 *DOI: 10.1515/aoas-2015-0074*

DE GRUYTER
OPEN

ANIMAL SPECIES IDENTIFICATION THROUGH HIGH RESOLUTION MELTING REAL TIME PCR (HRM) OF THE MITOCHONDRIAL 16S RRNA GENE*

Pitchayanipa Klomtong[1], Yupin Phasuk[1], Monchai Duangjinda[1,2♦]

[1]Department of Animal Science, Faculty of Agriculture,
[2]Research and Development Network Center for Animal Breeding (NCAB), Faculty of Agriculture,
Khon Kaen University, Khon Kaen, 40002, Thailand
♦Corresponding author: monchai@kku.ac.th

Abstract

Animal species identification has received growing attention, regarding genetic diversity and food traceability. The objective of this study is to apply a universal primer of part of the mitochondrial 16S rRNA gene analysis using the PCR-RFLP and HRM methods for identification of species origin in cattle, chicken, horse, sheep, pig, buffalo, and goat. PCR product size was 512 bp. The PCR product of 16S rRNA was digested with two restriction enzymes (*Bcl*I and *Mse*I); sufficient to easily generate analyzable species-specific restriction profiles that could distinguish the unambiguity of all targeted species. The HRM method successfully identified all species by shape of melting temperature, and proved to be of higher resolution, and a more cost effective, alternative method compared with other identification techniques.

Key words: 16S rRNA gene, HRM, PCR-RFLP, species identification

Species identification is important for genetic classification, animal biodiversity, and is becoming increasingly important in food traceability (Weitschek et al., 2014; Loftus, 2005). Several methods are used to identify DNA variation between species including single-strand conformational polymorphism (Gasser et al., 1998), random amplified polymorphic DNA (RAPD) (Shioda et al., 2003), amplified fragment length polymorphism (Gupta et al., 2004), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)**,** DNA sequencing, as well as conventional real

*Source of research financing: This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, and the Office of the Higher Education Commission, through the Food and Functional Food Research Cluster of Khon Kaen University.

time PCR (Dalvit et al., 2007). However, these methods are time-consuming and high-cost (Hellberg and Morrissey et al., 2011; Krenkova et al., 2009). PCR-RFLP has been determined to be one of the most efficient identification methods in terms of cost, detection power, and applicability to large scale screening, and as an alternative to direct DNA sequencing of PCR products. Interestingly, recent next-generation of Real time PCR techniques, such as the High Resolution Melting analysis (HRM) as powerful method, measure the rate of double stranded DNA dissociation to single stranded DNA, with increasing melting temperatures (Reed and Wittwer, 2004). HRM also has the presence of a third generation fluorescent dsDNA dye, present in analyses involving the scanning of single nucleotide polymorphisms (SNPs) and genotypes; as applied to species identification of plants, and bacteria; as well as the detection of meat adulteration (Ganopoulos et al., 2012, 2011; Madesis et al., 2012).

Mitochondrial DNA (mtDNA) is the tool most frequently used for identification in animal species; due to the high copy number per cell and high mutation rate, which impacts sequence variation among closely-related species (Pakendorf and Stoneking, 2005; Girish et al., 2004; Dooley et al., 2004). Species identification involving the mitochondria region generally includes the Cytochrome b, 12S rRNA, Cytochrome oxidase I (*COI*), D-loop, and 16S rRNA genes (Bravi et al., 2004; Girish et al., 2005; Haider et al., 2012; Haunshi et al., 2009; Mitani et al., 2009). At this time, Cytochrome oxidase subunit I (*COI*) is a marker for species identification, such as eutherian mammals, chicken, bacteria, amphibians and Mollusca (Hebert et al., 2003; Vences et al., 2005; Feng et al., 2011). Although the *COI* is the popular gene for animal species identification with high efficiency and specificity, only PCR-RFLP methods from large fragment (710-bp) could be applied in cow, chicken, turkey, sheep, pig, buffalo, camel and donkey (Haider et al., 2012). The complement of 16S rRNA to *COI* gene has been reported in amphibians and molluscs (Vences et al., 2005; Feng et al., 2011). In addition, higher variation of 16S rRNA gene has also been reported in Huagui Zhikong scallop by Yuan et al. (2009). Yang et al. (2014) also reported that the *rRNA* genes shared the homologous structures in species of organisms, ranging from bacteria to humans, due to the fact that numerous nucleotides are highly similar whereas such genes exhibit inter- and intra-specific nucleotide variations. In livestock, few publications of 16S rRNA gene have been reported (Mitani et al., 2009; Sarri et al., 2014). Therefore, in this study, we intend to demonstrate the HRM methods as screening tool for identification of seven meat animal species (cattle, chicken, horse, sheep, pig, buffalo, and goat) based on the developed universal primers of the mitochondrial 16S rRNA gene.

Material and methods

Samples and DNA extraction

Genomic DNA was extracted from the blood of seven species: cattle (*Bos taurus*), chicken (*Gallus gallus*), horse (*Equus caballus*), sheep (*Ovis aries*), pig (*Sus scrofa*), buffalo (*Bubalus bubalis*), and goat (*Capra hircus*), using a guanidine hy-

drochloride protocol modified from Goodwin et al. (2007). The extracted DNA was quantified using a Nano Drop (ND) 2000 spectrophotometer (Nano Drop Thermo Scientific, Wilmington, DE) and stored at -20°C for use in subsequent amplification.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

The design of primers and finding restriction enzyme specific for mutation site that allowed the discrimination between species were carried out for the first and second steps. First, the complete mitochondrial sequences of seven species: 1) V00654.1 (cattle, *Bos taurus*), 2) NC_005044 (goat, *Capra hircus*), 3) NC_001941 (sheep, *Ovis aries*), 4) GU147934 (pig, *Sus scrofa*), 5) HQ439493.1 (horse, *Equus caballus*), 6) AP003317.1 (chicken, *Gallus gallus*), and 7) NC_006295.1 (buffalo, *Bubalus bubalis*) from Genbank (http://www.ncbi.nlm.nih.gov/genbank) were used (Clustalw2 software) to identify regions of similarity and difference between seven species for selected primer region, to design the universal primer for 16S rRNA gene by Primer Blast program (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Second, identification of restriction enzyme allowing mutant discrimination by in-silico analysis was performed using the NEBcutter V2 program (Vincze et al., 2003).

The PCR was performed with 10 µl containing 10 ng of DNA 1µl, 1µl of 10x PCR buffer, 1µl of 3µM of primers in 16S rRNA. The universal primer for the 16S rRNA gene was forward (5'-TGACCGTGCAAAGGTAGCAT-3'), reverse (5'- ACCTGGATTACTCCGGTCTGA -3'), then amplified to the fragment size of 512 bp, 1µl of 10mM of 10 µM dNTP, 0.8µl 50mM $MgCl_2$, and 0.1µl of 5 U Taq DNA polymerase (MBI Fermentas). Using a TProfessional Standard Gradient Biometra (Germany), these reactions were amplified under the following conditions: five minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at the annealing temperature of 62°C, 30 seconds at 72°C, and a final extension of five minutes at 72°C.

The digestion of restriction enzymes (*Bcl*I and *Mse*I, New England Bio Lab, USA) was performed in a total volume of 10µl, containing 3µl of PCR product, 1U of restriction enzymes, 1µl of a digestion buffer, and sterile water was added up to 10 µl. The products were then incubated at 50°C and 37°C, respectively, overnight. The digested products were visualized by electrophoresis on 6% polyacrylamide gel and stained with gel star (Lonza, USA). Electrophoresis ran at 100 volts for 40 minutes at room temperature.

High Resolution Melting Analysis

The HRM analyses used the CFX96 Touch™ Real time PCR Detection System. The total volume of 25µl contained 2µl of genomic DNA/species, and 12.5µl of SsoFast™ EvaGreen® Supermix (containing dNTPs, Sso7d fusion polymerase, $MgCl_2$, EvaGreen® dye, and stabilizers). The melting curve analysis used CFX 2-step Amp-EvaGreen® melting conditions; consisting of one cycle with initial denaturation at 95°C for 10 minutes, 35 cycles starting denaturation at 95°C for 15 seconds, and underwent annealing at 61°C for 20 seconds. Readings were taken every cycle. The threshold level was fixed at 500 -d (F)/dT throughout all samples of the experiment.

The melting curve of PCR products, seen on the monitor, showed that the fluorescence values were plotted at slow ramp (0.2°C) intervals from 65 to 95°C. Changes in fluorescence of EvaGreen® during PCR and the melting phase were monitored on the SYRB green channel of CFX manager software. Afterwards, HRM used Precision Melt Analysis™ and Software BioRad® to detect different species by clustering number. All samples were examined in duplicate.

Specificity and sensitivity test

To confirm assay specificity, 10 ng of genomic DNA from seven species: cattle (n=20), chicken (n=10), horse (n=3), sheep (n=15), pig (n=20), buffalo (n=20), and goat (n=20)) were used to amplify with HRM real time PCR method and to detect RFLP patterns by PCR-RFLP assay. No amplification product was obtained when without genomic DNA. The shape of curve from HRM and RFLP pattern of DNA from all seven species must be consistent to show species specificity. In sensitivity tests, DNA samples of seven species were diluted to 10 ng in sterile water and then serial dilutions were made down to 1 in 1×10^6 to determine the sensitivity of the PCR assay. The standard curves for both detection systems were constructed by using the cq value obtained from the corresponding DNA concentration.

Results

Variation of 16S rRNA gene in seven animal species by PCR-RFLP method

A fragment of the mitochondrial 16S rRNA gene of approximate size 512 bp was amplified from the samples of all seven species. There was no size variation among samples. A schematic figure of predicted RFLP patterns for specific differentiation of seven species with 16S rRNA gene was generated. *MseI* enzyme generated specific RFLP patterns for the differentiation of seven species: the RFLP patterns of cattle resulted in 195 bp, 159 bp, 80 bp, 43 bp, 28 bp and 7 bp, which were indistinguishable from the fragments observed for buffalo 196 bp, 159 bp, 115 bp and 43 bp; sheep fragment size was 195 bp, 159 bp, 62 bp, 53 bp and 43 bp, goat digestion pattern 196 bp, 130 bp, 87 bp, 80 bp, 43 bp, 29 bp and 28 bp, pig digestion pattern 174 bp, 159 bp, 63 bp, 47 bp, 43 bp, 23 bp and 4 bp, horse digestion fragment, 172 bp, 159 bp, 118 bp, 40 bp and 23 bp, chicken fragment pattern 302 bp, 89 bp, 70 bp, 43 bp and 41 bp (Figure 1 A).

Consequently, it was crucial to take into account this finding for experimental confirmation of predicted band patterns and we found that cattle, buffalo, sheep, goat and pig could be identified with Mse l, while chicken and horse showed similar pattern, and the pattern was unexpected in chicken (Figure 1 C) due to different genetic background of chicken samples used in this study. Thus, it was necessary to find the other enzyme which allows discriminating between chicken and horse; thus BciI enzyme generated exclusive band patterns based on polymorphic sequence (Figure 1 B). Six animals showed the same restriction patterns of *Bcl*I (328 bp and 184 bp), except that of the chicken which was undigested and showed the same result between

RFLP prediction and experiment, thus it could be discriminated between chicken and horse (Figure 1 D).

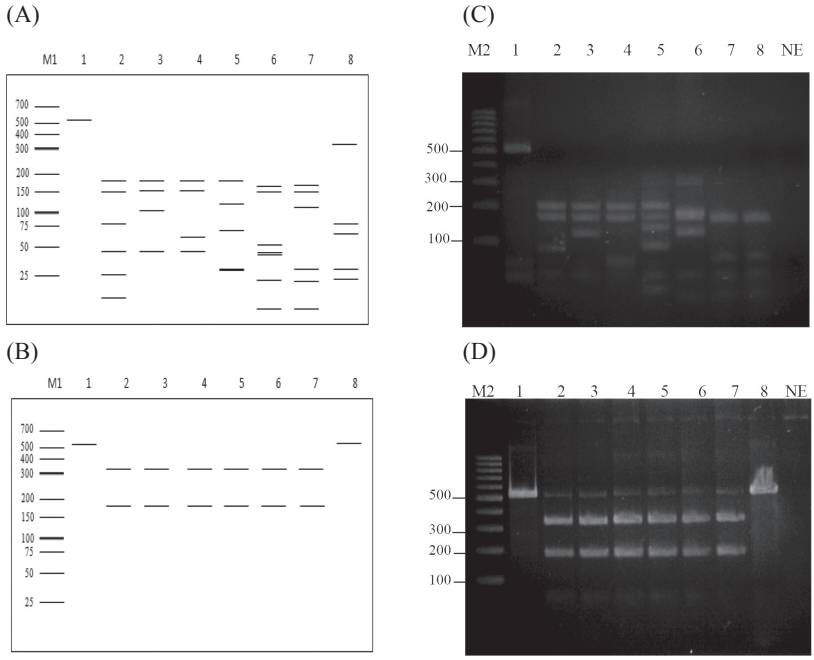
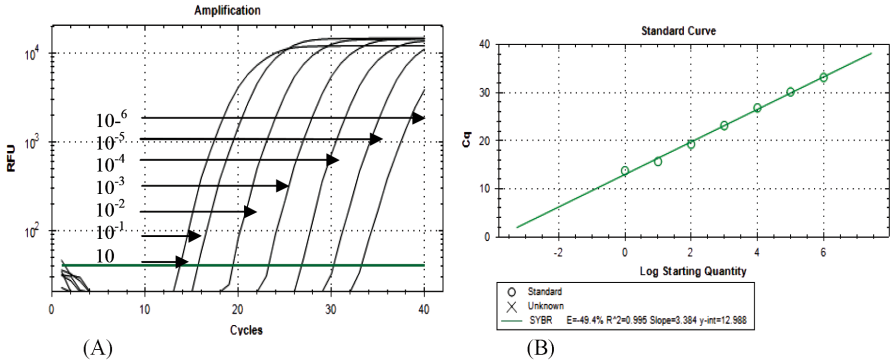


Figure 1. PCR-RFLP patterns of seven animal species obtained by digestion with *Mse*I and *Bcl*I using 16S rRNA gene. Lane: M1, DNA ladder (50 bp ladder); Lane: M2, DNA ladder (100 bp ladder); 1, PCR product size; 2, cattle (*Bos taurus*); 3, buffalo (*Bubalus bubalis*); 4, goat (*Capra hircus)*; 5, sheep (*Ovis aries)*; 6, pig (*Sus scrofa*); 7, horse (*Equus caballus*), and 8, chicken (*Gallus gallus*). NE refers to negative control (without genomic DNA). (A) and (B) *In silico* analysis and (C) and (D), 2% agarose gel electrophoresis with *Mse*I and *Bcl*I, respectively

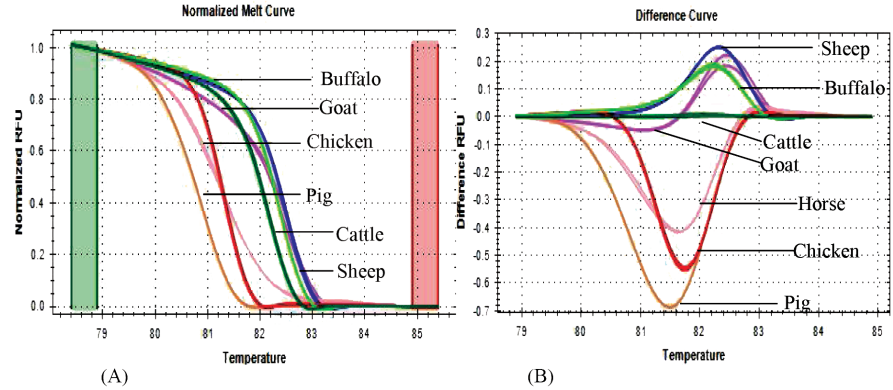
High Resolution Melting Analysis for identification of animal species

The sensitivity and linear regression of the 16S rRNA gene by EvaGreen® dye were detected using ten-fold serial dilutions of the genomic DNA obtained from each of species, starting with 10 ng to 0.01 pg. The standard curve with the Cq values was plotted against the initial quantity of DNA, to test linear regression. The correlation between the Cq value and the logarithm of the target DNA concentrations of cattle showed a determination of coefficient values of 0.995, as shown in Figure 2. The HRM assay, a fragment of the mitochondrial 16S rRNA gene of approximate size 512 bp, was amplified from the samples of all species. Normalized melt curves were sufficient to identify the sequence variation with melting curve shapes after clustering the Bio Rad software that precision melting analysis indicates the following melting temperatures: cattle 82.2°C±0.2, goat 82.6°C±0.2, pig 81.2°C±0.2, chicken 81.4°C±0.2, horse 81.2°C±0.2, buffalo 82.4°C±0.2, and sheep 82.4°C±0.2, and when we analyzed by difference plot curve showed the same single curve shape, as shown in Figure 3.



(A) (B)

Figure 2. (A) Quantification of ten-fold dilution series of cattle DNA. (B) Standard curve showing the Cq values in relation to the concentration of initial amounts of DNA, obtained by a serial ten-fold dilution of cattle species (from 10 ng to 0.01 pg DNA)



(A) (B)

Figure 3. HRM assay use for identification of animal species with the 16S rRNA gene. (A) is the normalized melt curve, and (B) the difference plot curve

Discussion

16S rRNA is a ribosomal RNA encoded by the mitochondrial genome, necessary for the translation of messenger RNA into mitochondrial proteins. The 16S rRNA gene is about 1,570 bp long (Chen et al., 2013). Despite its diminutive size, the gene is used as a genetic marker to identify mammals, poultry, and invertebrates; using universal primer sets (Mitani et al., 2009; Sarri et al., 2014; Yang et al., 2014). The 16S rRNA gene harbors a larger number of species-specific mutation sites than the cytochrome b gene (Guha et al., 2006). With two minimum enzymes *Bcl*I, and *Mse*I, we employed endonuclease for RFLP analysis to identify seven common livestock animals. Additionally, the PCR-RFLP of the 16S rRNA gene successfully differentiated closely related meat species, such as cattle with buffalo, and sheep with goats.

Compared with other molecular methods, such as Terminal Restriction Fragment Length Polymorphism (T-RFLP) and DNA sequencing (Mitani et al., 2009; Wang et al., 2010), the PCR-RFLP procedure established in this study may be easily conducted in a laboratory, without the requirement of high-standard equipment, in a more cost effective manner. Although the PCR-RFLP method is accurate and repeatable, it is both time consuming and overly expensive, when the digested PCR products are separated by gel electrophoresis.

HRM as a tool for disclosure, is an extension of melting curve analysis (MCA), and employs next generation Real time PCR thermo cyclers with high thermal and optical precision (Vezenegho et al., 2009). HRM provides a powerful approach much greater than conventional melting curve analysis, as it can differentiate the PCR product's basic shape of melting temperatures when defining the same melting temperature (Ganopoulos et al., 2012). MCA is unable to make distinctions at melt peak, but HRM analysis can dissociate the products based on their shape due to the differentiation of mutation types, such as SNPs and the presence of insertions or deletions (indels) (Ganopoulos et al., 2012). A report by Santos et al. (2012) which used the EvaGreen® dye for monoplex primer hare identification, established a highly specific technique using 1 pg.

The HRM assay can discern a single nucleotide polymorphism or other mutation by comparing the relative position and shape of the melt curves in the presence of a third generation fluorescent, dsDNA dye. Prior to this time, HRM was connected to fluorescent dyes, such as LC Green, ResoLight®, EvaGreen® (EG), and SYTO9. EG is intercalating and dye-based, and used for quantitative Real time PCR (qPCR), melt curve analysis (MCA), as well as HRM (Santos et al., 2012; Mao et al., 2007; Sakaridis et al., 2013). Mao et al. (2007) found that when comparing intercalating dyes (such as EG and SYBR Green I), EG had good characterization, containing the widely used qPCR dye DNA-binding properties and stability. However, when using intercalating dyes, we must authenticate the target species, due to the non-specific fluorescent signal. In addition, the results were affected by the sequence of the sample, generating heteroduplex from random concentration of Mg^{2+} and other chemical variable (Reja et al., 2010).

In other studies, Ganopoulos et al (2013) used specific mitochondrial DNA regions with high resolution melting (HRM) to detect bovine, ovine, and caprine species; through an attempt to authenticate Greek PDO Feta cheese. The primers successfully amplified DNA isolated from milk and cheese, and showed a high degree of specificity. HRM was proven capable of accurately identifying the presence of bovine milk within 0.1%, as well as quantifying the ratio of sheep to goat milk mixture in different commercial Feta cheese products. Sakaridis et al. (2013) reported that the identification of meat samples from five animal species (buffalo, bovine, pork, sheep, and goat), may be distinguished using HRM analysis (melting profiles of the 18S rRNA amplicons) using universal primers (18S rRNA). Ganopoulos et al. (2011) and Madesis et al. (2012) reported that the application of the HRM analysis method of universal plant DNA, employing regional barcoding via microsatellite, permitted the identification, adulteration and quantification, of Basmati rice and general bean species.

This method offers high sensitivity and is widely applicable. Through mitochondrial DNA amplification, degraded (or small) amounts of forensic samples or meat products can be analyzed. The universal primer set enables PCR amplification of the 16S rRNA sequences of samples from many animal species, incorporating HRM methods which prove simple, fast, and cost effective.

In conclusion, we have demonstrated that both PCR-RFLP and HRM are useful methods of identifying the relative proximity of several closely related commercially important animal species. The use of designed universal 16S rRNA primer was added to successfully identify seven animal species, using an HRM method that could be effectively applied to species identification tests in routine control methods.

References

Bravi C.M., Lirón J.P., Mirol P.M., Ripoli M.V., Peral-García P., Giovambattista G. (2004). A simple method for domestic animal identification in Argentina using PCR-RFLP analysis of cytochrome b gene. Leg. Med. (Tokyo, Japan), 6: 246–251.

Chen C.H., Huang H.L., Yang H.Y., Lai S.H., Yen N.T., Wu M.C., Huang M.C. (2013). Mitochondrial genome of Taiwan pig (*Sus scrofa*). Afr. J. Biotechnol., 10: 2556–2561.

Dalvit C., De Marchi M., Cassandro M. (2007). Genetic traceability of livestock products: A review. Meat Sci., 77: 437–449.

Dooley J.J., Paine K.E., Garrett S.D., Brown H.M. (2004). Detection of meat species using TaqMan real-time PCR assays. Meat sci., 68: 431–438.

Feng Y., Li Q., Kong L., Zheng X. (2011). DNA barcoding and phylogenetic analysis of Pectinidae (Mollusca: Bivalvia) based on mitochondrial *COI* and 16S rRNA genes. Mol. Biol. Rep., 38: 291–299.

Ganopoulos I., Argiriou A., Tsaftaris A. (2011). Microsatellite high resolution melting (SSR-HRM) analysis for authenticity testing of protected designation of origin (PDO) sweet cherry products. Food Control., 22: 532–541.

Ganopoulos I., Madesis P., Tsaftaris A. (2012). Universal ITS2 barcoding DNA region coupled with high-resolution melting (HRM) analysis for seed authentication and adulteration testing in leguminous forage and pasture species. Plant. Mol. Biol. Report., 30: 1322–1328.

Ganopoulos I., Sakaridis I., Argiriou A., Madesis P., Tsaftaris A. (2013). A novel closed-tube method based on high resolution melting (HRM) analysis for authenticity testing and quantitative detection in Greek PDO Feta cheese. Food Chem., 141: 835–840.

Gasser R.B., Monti J.R., Bao-Zhen Q., Polderman A.M., Nansen P., Chilton N.B. (1998). A mutation scanning approach for the identification of hookworm species and analysis of population variation. Mol. Biochem. Parasitol., 92: 303–312.

Girish P.S., Anjaneyulu A.S.R., Viswas K.N., Anand M., Rajkumar N., Shivakumar B.M., Bhaskar S. (2004). Sequence analysis of mitochondrial 12S rRNA gene can identify meat species. Meat Sci., 66: 551–556.

Girish P.S., Anjaneyulu A.S.R., Viswas K.N., Shivakumar B.M., Anand M., Patel M., Sharma B. (2005). Meat species identification by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of mitochondrial 12S rRNA gene. Meat Sci., 70: 107–112.

Goodwin W., Linacre A., Hadi S. (2007). An introduction to forensic genetics. John Wiley&Sons Ltd., Chichester, England.

Guha S., Goyal S.P., Kashyap V.K. (2006). Genomic variation in the mitochondrially encoded cytochrome b (MT-CYB) and 16S rRNA (MT-RNR2) genes: characterization of eight endangered Pecoran species. Anim. Genet., 37: 262–265.

Gupta A.K., Boekhout T., Theelen B., Summerbell R., Batra R. (2004). Identification and typing of Malassezia species by amplified fragment length polymorphism and sequence analyses of the internal transcribed spacer and large-subunit regions of ribosomal DNA. J. Clin. Microbiol., 42: 4253–4260.

Haider N., Nabulsi I., Al-Safadi B. (2012). Identification of meat species by PCR-RFLP of the mitochondrial COI gene. Meat Sci., 90: 490–493.

Haunshi S., Basumatary R., Girish P.S., Doley S., Bardoloi R.K., Kumar A. (2009). Identification of chicken, duck, pigeon and pig meat by species-specific markers of mitochondrial origin. Meat Sci., 83: 454–459.

Hebert P.D.N., Ratnasingham S., deWaard J.R. (2003). Barcoding animal life: cytochrome coxidase subunit 1 divergences among closely related species. P. Roy. Soc. B-Biol. Sci., 270: S96–S99.

Hellberg R.S.R., Morrissey M.T. (2011). Advances in DNA-based techniques for the detection of seafood species substitution on the commercial market. J. Assoc. Lab. Autom., 16: 308–321.

Krenková P., Norambuena P., Stambergová A., Macek M. (2009). Evaluation of high-resolution melting (HRM) for mutation scanning of selected exons of the CFTR gene. Folia Biol. (Praha), 55: 238–242.

Loftus R. (2005). Traceability of biotech-derived animals: application of DNA technology. Rev. Sci. Tech. O.I.E., 24: 231–242.

Madesis P., Ganopoulos I., Anagnostis A., Tsaftaris A. (2012). The application of Bar-HRM (Barcode DNA-High Resolution Melting) analysis for authenticity testing and quantitative detection of bean crops (Leguminosae) without prior DNA purification. Food Control., 25: 576–582.

Mao F., Leung W.Y., Xin X. (2007). Characterization of EvaGreen and the implication of its physicochemical properties for qPCR applications. BMC Biotechnol., 7: 76.

Mitani T., Akane A., Tokiyasu T., Yoshimura S., Okii Y., Yoshida M. (2009). Identification of animal species using the partial sequences in the mitochondrial 16S rRNA gene. Leg. Med. (Tokyo, Japan), 11 Suppl 1: S449–450.

Pakendorf B., Stoneking M. (2005). Mitochondrial DNA and human evolution. Annu. Rev. Genom. Hum. G., 6: 165–183.

Reed G.H., Wittwer C.T. (2004). Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. Clin. Chem., 50: 1748–1754.

Reja V., Kwok A., Stone G., Yang L., Missel A., Menzel C., Bassam B. (2010). ScreenClust: Advanced statistical software for supervised and unsupervised high resolution melting (HRM) analysis. Methods, 50: S10–S14.

Sakaridis I., Ganopoulos I., Argiriou A., Tsaftaris A. (2013). A fast and accurate method for controlling the correct labeling of products containing buffalo meat using High Resolution Melting (HRM) analysis. Meat Sci., 94: 84–88.

Santos C.G., Melo V.S., Amaral J.S., Estevinho L., Oliveira M.B.P.P., Mafra I. (2012). Identification of hare meat by a species-specific marker of mitochondrial origin. Meat Sci., 90: 836–841.

Sarri C., Stamatis C., Sarafidou T., Galara I., Godosopoulos V., Kolovos M., Liakou C., Tastsoglou S., Mamuris Z. (2014). A new set of 16S rRNA universal primers for identification of animal species. Food Control., 43: 35–41.

Shioda H., Satoh K., Nagai F., Okubo T., Seto T., Hamano T., Kamimura H., Kano I. (2003). Identification of Aloe species by random amplified polymorphic DNA (RAPD) analysis (in Japanese). Shokuhin Eiseigaku Zasshi, 44: 203–207.

Vences M., Thomas M., van der Meijden A., Chiari Y., Vieites D.R. (2005). Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. Front. Zool., 2: 5.

Vezenegho S.B., Bass C., Puinean M., Williamson M.S., Field L.M., Coetzee M., Koekemoer L.L. (2009). Development of multiplex real-time PCR assays for identification of members of the *Anopheles funestus* species group. Malaria J., 8: 282.

Vincze T., Posfai J., Roberts R.J. (2003). NEBcutter: a program to cleave DNA with restriction enzymes. Nucleic Acids Res., 31: 3688–3691.

Wang Q., Zhang X., Zhang H.Y., Zhang J., Chen G-Q., Zhao D-H., Ma H-P., Liao W-J.

(2010). Identification of 12 animal species meat by T-RFLP on the 12S rRNA gene. Meat Sci., 85: 265–269.

Weitschek E., Fiscon G., Felici G. (2014). Supervised DNA Barcodes species classification: analysis, comparisons and results. BioData Min., 7: 4.

Yang L., Tan Z., Wang D., Xue L., Guan M., Huang T., Li R. (2014). Species identification through mitochondrial rRNA genetic analysis. Sci. Rep., 4.

Yuan T., He M., Huang L. (2009). Intraspecific genetic variation in mitochondrial 16S rRNA and COI genes in domestic and wild populations of Huaguizhikong scallop *Chlamys nobilis* Reeve. Aquaculture, 289: 19–25.

Received: 12 V 2015
Accepted: 27 X 2015