A suitable method for the detection of a potential fraud of bringing macaque monkey meat into the food chain

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Accepted author version posted online: 23 Apr 2015. Published online: 07 May 2015.


To link to this article: http://dx.doi.org/10.1080/19440049.2015.1039073

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A suitable method for the detection of a potential fraud of bringing macaque monkey meat into the food chain


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(Received 12 March 2015; accepted 5 April 2015)

Being the third-largest primate population has not made macaque (Macaca fascicularis sp.) monkeys less exposed to threats and dangers. Despite wildlife protection, they have been widely hunted and consumed in several countries because of their purported nutritional values. In addition to trading as pure bush meats in several places, monkey meat has been sold in meatball and soup products in Indonesia. Thus the possibility of macaque meat trafficking under the label of common meats is quite high. This paper reports the development of a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay with the shortest amplicon length for the confirmed detection of monkey meat under compromised states which are known to degrade DNA. We amplified a 120-bp region of d-loop gene using a pair of macaque-specific primers and confirmed their specificity for the target species through cross-challenging against 17 different species using a 141-bp site of an 18 S rRNA gene as an endogenous control for eukaryotes. This eliminated the possibilities of any false-negative detection with complex matrices or degraded specimens. The detection limit was 0.00001 ng DNA in a pure state and 0.1% of meat in mixed matrices and commercial meatball products. RFLP analysis further authenticated the originality of the PCR product and distinctive restriction patterns were found upon Alu and CViki-1 digestion. A micro-fluidic lab-on-a-chip automated electrophoretic system separated the fragments with high resolution. The assay was validated for screening commercial meatball products with sufficient internal control.

Keywords: macaque monkey; meat trafficking; compromised states; restriction patterns; endogenous control; reproducibility

Introduction

Wild meat has great appeal to exotic lovers and elites because of its exquisite taste and healthier attributes of more proteins and less fat (Hoffman & Cawthorn 2012). It has been believed that exotic meats could increase internal energy, giving longer life and increase juvenility and youth hood (Hoffman & Cawthorn 2012). South Africa, Australia, Europe and America are the major producers of game meat such as deer, kangaroo and ostrich and, hence, these animals are reared on farms for meat (Hoffman & Wiklund 2006). However, an insufficient domestic supply and overpricing of red meat in some developing countries might push consumers to hunt for their own animal proteins in natural habitats such as forests and bushes (Hoffman & Cawthorn 2012). An estimated wild meat harvest in Central Africa is 3.4 million tons per annum (Bennett 2002). Southeast Asia, Malaysia, Indonesia, Philippines and Cambodia are the main exporters of wild meat naturally grown in their vast rainforests.

A variety of different wildlife species including primates have remained a cheap source of proteins for many population groups. Long-tailed macaques (Macaca fascicularis) have been enlisted as the species of least concern by the International Union for Conservation of Nature (IUCN) Redlist (Ong & Richardson, 2008). However, their population is decreasing due to the large amount of hunting for both consumption and research (Eudey 2008). More than 5.5 million primate specimens were traded legally or illegally from 1990 to 2004 according to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) trade database (see www.cites.org) (Rönn et al. 2009). According to the published report, illegal trade represent less than 1% of that number. However, we believe this percentage and the total number of specimens traded are an underestimation since most of the illegal trade remains undocumented. The excessive harvesting of wild animals for meat and the concomitant decline in many species represents a major threat both to both biodiversity and people’s livelihoods (Hoffman & Cawthorn 2012).

In 2010, a report was aired on ABC News that monkey meat was used in meatball and soup products in Indonesia, and a couple was arrested for it (Creagh 2010); recently The Times of India reported the rampant...
trade of monkey meat in certain regions of India for the export of monkey meat and brain to Africa, Japan, Korea, Taiwan, China and other countries through certain agencies (Drolia 2014). Since macaque is a widespread species and often can be hunted free of charge, the chances of macaque meat falsification is very high. The great concern for species threats, illegal trades and meat fraudulence effects on consumers’ health and religious faith forced us to develop a suitable meat speciation technique especially for wild meat such as macaque meats. Wild animals, especially those that are caught in the wild, have a great tendency to be associated with pathogenic zoonosis bacteria (Escherichia coli, Salmonella sp., Mycobacterium tuberculosis) and viruses (such as avian influenza and simian immunodeficiency virus – SIV) (Chomel et al. 2007; Ramanzin et al. 2010).

In meat speciation, DNA-based techniques have been preferred over protein and lipid-based molecular identification schemes since DNA biomarkers, especially the short-length ones, are extremely stable even under harsh processing condition (heat, pressure and chemical additives) as well as compromised states such as natural decomposition or degraded specimens where protein-based markers are denatured or degraded and lipid-based biomarkers can be made rancid (Fajardo et al., 2010). Mitochondrial DNAs (mtDNA) or genes are especially suitable for meat speciation applications since they are found in multiple copies in each cell with adequate polymorphisms or species-specific fingerprints (Murugaiah et al. 2009).

On the other hand, PCR-based detection schemes are amazing since they are simple, cost-effective and robust and can amplify marker DNA targets even from a single or a few copies to detectable quantities (Verkaar et al. 2002; Ong et al. 2007). Species-specific PCR (Che Man et al. 2007; Haunshi et al. 2009; Mane et al. 2012; Karabasanavar et al. 2014), multiplex PCR (Dooley et al., 2004; Ali et al. 2015) randomly amplified polymorphic DNA (RAPD) (Arslan et al. 2006), PCR restriction fragment length polymorphism (RFLP) (Ali, Hashim, Mustafa, Che Man 2012), and real-time PCR (Ali, Hashim, Mustafa, Che Man, Dhahi, et al. 2012) are some of the significant reports for the identification of meat species.

Species-specific PCR-RFLP assays are advantageous since they not only amplify-specific targets but also authenticate whether real-targets are detected through a post-PCR restriction digestion (Ali, Hashim, Mustafa, Che Man 2012). They have special interest in meat speciation because they exploit the sequence variations that exist within a defined region of target DNA, allowing differentiation of even closely related species by digestion of selected DNA fragments with appropriate restriction enzymes (Fajardo et al. 2008). PCR-RFLP assays have been documented to distinguish between the closest species such as cattle–buffalo and sheep–goat (Girish et al. 2005), swine and wild boar (Fajardo et al. 2008), various fish species (Wolf et al. 2000), and cattle and yak (Chen et al. 2010). However, no PCR-RFLP assay has been made in public for macaque meat speciation. Here we successfully amplified a very short (120 bp) target of d-loop gene using macaque-specific primers, digested with two different restriction enzymes (AluI and CVI1-1) and obtained distinctive identifying restriction fingerprints for macaque species.

Materials and methods

Collection of meat samples

All raw meat samples (chicken, beef, buffalo, goat, lamb, duck, pork, venison, carp, cod and salmon) were purchased in triplicate from various markets in Selangor (Pasar Borong Jalan Othman, Petaling Jaya and Pasar Borong Selangor, Serdang) as well as AEON Supermarkets in Kuala Lumpur, Malaysia. Other species, such as quail, pigeon and turtle, were bought from Pudu Wet Market, Kuala Lumpur. Three different cat and dog meat samples were collected after being euthanised by the authorised personnel of Dewan Bandaraya Kuala Lumpur, Malaysia. Meanwhile, the target species, macaque meat samples from three different Macaca fascicularis sp. were provided for study purpose by the Department of Wildlife and National Parks (DWNP) Peninsular Malaysia (Cheras, Kuala Lumpur). For commercial meatballs, a total of eight different brands of beef and chicken meatball (four brands for each) were bought from different stores in Mid Valley Megamall, Kuala Lumpur. All samples were stored at −20°C until further use to prevent enzymatic degradation of DNA.

Monkey-specific primer

Macaca fascicularis mt-d-loop gene sequence (FJ906803.1) was retrieved from National Centre of Biotechnology Information (NCBI) and in silico designed using the publicly available software Primer3Plus (see http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). A pair of primers (forward primer: 5'-TGAAATCAATATCCCGCACA-3' and reverse primer: 5'-CTGGTTGTATGCGGCTGAG-3') targeting a 120-bp fragment of d-loop gene of monkey were selected. The specificity of the primers was tested in three different ways. Firstly, online Basic Local Alignment Tool (BLAST) against non-redundant nucleic acid sequences in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to screen the identical and distant species. Secondly, the primers were multiple aligned with 17 other species – cow (Bos taurus): AB003801.1, sheep (Ovis aries): AB003801.1, red fox (Vulpes vulpes): AB003801.1, wild horse (Equus caballus): AB003801.1, giant panda (Ailuropoda melanoleuca): AB003801.1, Japanese macaque (Macaca fuscata): AB003801.1, Siberian tiger (Panthera tigris altaica): AB003801.1, domestic pig (Sus scrofa domestica): AB003801.1, hog deer (Axis porcinus): AB003801.1, grey wolf (Canis lupus): AB003801.1, red fox: AB003801.1, yellow fox (Vulpes vulpes): AB003801.1, brown bear (Ursus arctos): AB003801.1, striped hyena (Hyaena hyaena): AB003801.1, red fox: AB003801.1, and striped hyena: AB003801.1. The results of the in silico design using Primer3Plus were compared with the in silico design using Primer3Plus and the results were in accordance with the in silico design using Primer3Plus.
**DNA extraction and PCR amplification**

Total DNA was extracted from 30 mg of each type of meat sample (raw, admixed and commercial meatballs) using DNA Mini Kit for Animal Tissues (Yeaster Biotech Co. Ltd, Taipei, Taiwan) following the manufacturer’s instruction without any modification. Concentration and purity were checked by a UV-vis spectrophotometer (Biochrom Libra S70, Biochrom Ltd, Taipei, Taiwan) taking absorbance at 260–280 nm.

Target DNA was amplified in a 250 µl PCR tubes in 20 µl reaction mixture containing 4 µl of 5× Green GoTaq Flexi Buffer, 2.2 µl of 25 mM of MgCl₂, 0.4 µl of 0.2 mM of each DNTP, 0.4 µM of each primers and 0.5 unit Taq polymerase and 10 ng of total DNA extracted from each sample. We also included 0.4 µM of eukaryotic 18 S rRNA primers (forward primer: GGT AGT GAC GAA AAA TAA CAA TAC AGC TGG AAT TAC C) internal control (Rojas et al. 2010; Ali, Hashim, Mustafa, Che Man 2012). Meanwhile, the negative control was set with nuclease-free distilled water to eliminate contamination. The primer set were purchased from First Base Laboratories Sdn. Bhd. (Selangor, Malaysia) and PCR reagents from Promega Corporation (Madison, WI, USA). The PCR reaction was performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems Inc., Foster, CA, USA), following initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 1 min and the final extension at 72°C for 5 min. PCR products were kept at ~20°C for further analysis.

**Assay sensitivity in pure and admixed meats**

The sensitivity was tested by dilution of DNA extracted from pure and admixed meats. LOD was determined by amplification of 10-fold serially diluted (10, 1, 0.1, 0.01, 0.001 and 0.0001 ng DNA) monkey DNA. For a binary mixture, two types of admixed were prepared: monkey–beef and monkey–goat mixtures in a total of 100 g specimens by spiking monkey meats at a proportion of 10%, 5%, 1% and 0.1%. The meats were first minced and then homogenised in a blender. To avoid contamination, each mixture was prepared separately using different material and different blender containers on three different days.

**Enzymatic digestion and RFLP analysis**

PCR products were digested with AluI and CVIKI-1 restriction endonucleases (New England Biolabs, Ipswich, MA, USA) in a 25 µl reaction mixture in separate tubes containing 1 µg of unpurified PCR product, 5 U of enzyme, 1× digestion buffer and an adjusted amount of sterilised distilled water. Digestion was carried out at 37°C in a shaking water bath for 45 min. After 45 min, AluI digestion was stopped by heating the mixture at 65°C for 20 min. However, no enzymatic inactivation steps were required for CVIKI-1 enzymes. For RFLP analysis, 1 µl digested product was applied to a microfluidic-lab-on-a-chip using 1 k DNA kit and was separated by Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA, USA).

**Commercial meatball analysis**

A total of four different brands of each commercial chicken and beef meatball were cross-tested with monkey-specific primer in triplicate. To simulate the real adulteration of meatball products in the market, we spiked monkey meat in commercial meatballs up to the detection limit of 0.1%. Eukaryotic endogenous control (141-bp site of 18 S rRNA) was used in every test to determine the quality of DNA in meatballs as well as monkey DNA.

**Results and discussion**

**Specificity test**

Species-specific PCR assays have been widely used for the detection of various species due to their simplicity, low cost and robust results. The key factor in obtaining a highly specific assay is primer design with required species fingerprints. The presence of a single mismatch in the primer binding site may reduce the efficiency of a PCR assay or might lead to false or no detection (Wu et al. 2009). Designing primers with perfect matching with the specific target and multiple mismatches with non-target species would definitely increase the specificity of the
final assay, decreasing the chances of non-target amplification. We retrieved the mtDNA sequence of *M. fascicularis* (FJ906803.1) from the NCBI and designed a set of primers specifically to amplify a short fragment 120 bp of the d-loop region. Multiple alignments with 17 other meat species revealed perfect matching (100%) only with monkey d-loop region and scored 3–17 nt (15–85%) mismatching with non-target species (Figure 1). Pairwise distance (Figure 1a), 3D plot (Figure 1b) and number of primer mismatches (Figure 1c) obtained using the neighbour-joining method (Tamura et al., 2011) showed clear discrimination of the monkey primers from all other species (Figure 1b). The shortest distance was found between monkey and rat (0.33), and the highest was obtained between monkey and cod fish (2.91), reflecting the huge genetic distance and likelihood of cross-species amplification in a real PCR run. Finally, a practical PCR experiment amplified only the *M. fascicularis* species (Figure 2), confirming the theoretical finding that the assay is highly specific for monkey species only.

Four different PCR assays have been documented for the detection of monkey species, mainly for the purpose of phylogenetic studies. However, the targets for those assays (Md-Zain et al. 2010) (cytochrome c, 850 bp), (Abdul-Latiff et al., 2014) (cytochrome b, 383 bp), (Hayasaka et al. 1996) (mt-whole genome, 896 bp) and (Blancher et al., 2008) (d-loop, 590 bp) were very large, which easily break down during food processing treatments. Thus the previously documented assays might not be suitable for meat species detection in foods (Ali, Hashim, Mustafa, Che Man 2012; Ali et al. 2014). Meanwhile, Rönn et al. (2009) proposed a first-generation microarray system for the detection of various primates species targeting the epsilon globin (341 bp) and apolipoprotein B gene (550 bp) sequences to trace out wild meat trades. However, the latest studies appreciated short-amplicon-length PCR assays (amplicon size < 150 bp) targeting multi-copy mitochondrial genes for the detection of animal species in highly processed foods (Ali, Hashim, Mustafa, Che Man 2012; Ali et al. 2014). Therefore, we have documented here a 120-bp PCR-RFLP assay targeting the mt-d-loop gene for *M. fascicularis* detection in processed meats. To the best of our knowledge, such a short-amplicon-length PCR-RFLP method for macaque meat detection is the first reported in the literature.

**Limit of detection (LOD)**

To date, no detection technique for monkey species detection in foods have been tested and optimised. The currently available monkey-specific PCR assays (Hayasaka et al. 1996; Blancher et al., 2008; Md-Zain et al. 2010; Mab et al. 2014) are suitable for evolutionary, taxonomic and phylogenetic studies among the species. Since these studies were not tested for meat authentication, their LOD has remained undefined. A serial dilution (10, 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001 ng) of DNA extracted from pure raw meat by three independent analysts on three different days was used to determine sensitivity under raw and pure states. Previously, Che Man et al. (2012) and Karabasanavar et al. (2014) tested their assay sensitivity for pork DNA by a dilution method and detected as low as 0.001 ng DNA μl⁻¹. Here we clearly observed 120-bp PCR products from a 0.00001 ng macaque DNA template and thus we defined the LOD for this assay under raw and pure states (Figure 3).

**RFLP analysis**

We successfully amplified the 120-bp site of the mitochondrial d-loop gene of macaque monkey in the presence of a 141-bp universal eukaryotic site of 18 S rRNA as an internal control to evaluate the quality of the DNA used as well as to eliminate any false-negative detection (Ali, Hashim, Mustafa, Che Man 2012). Although, species-specific PCR assays are often conclusive, authentication of amplified PCR products definitely increases the reliability of the assay. Three different methods, namely restriction analysis with at least two restriction endonucleases, probe hybridisation and DNA sequencing, could verify authentic PCR products (Maede 2006). Probe hybridisation is interesting since it can detect multiple species simultaneously using more than two DNA probes in a single hybridisation reaction (labelled dyes) or separately. However, this procedure is laborious and requires high-quality DNA which is less feasible for heat/chemically treated DNA extracted from processed meats or meat products (Mafra et al. 2008). On the other hand, DNA sequencing is reliable but it is time consuming, requires expensive laboratory set-up and thus is not suitable for routine meat specification assessment (Lockley & Bardsley 2000; Girish et al. 2004). Cooked or processed samples with degraded DNA and complex food matrices might further complicate it, hindering result interpretation. In contrast, PCR-RFLP has been extensively used to distinguish two or more closest species with simple instrumentation (Verkaar et al. 2002; Ong et al. 2007). It comprises the generation of species-specific band profiles through restriction digestion with one or more restriction endonucleases (Pereira et al. 2008). These restriction enzymes cleave the DNA molecule at recognition sites, originating a set of fragments with different lengths that could be separated according to their molecular size by electrophoresis (Pereira et al. 2008). Thus, PCR-RFLP has been proved to be a practical, highly repeatable and reliable technique for meat species identification (Haider et al. 2012).

Here we digested the 120-bp monkey-specific PCR products by two different enzymes, AluI and CVIKI-1 since in
In silico analysis showed available restriction sites for these enzymes with suitable fragment lengths (New England Biolabs; see http://nc2.neb.com/NEBcutter2/). Two sites for AluI and four sites for CViKI-1 were found within the amplified sequence (120 bp) (Figure 1c). Lane 4 in Figure 4 demonstrates two fragments of length 65 and 44 bp, which

<table>
<thead>
<tr>
<th>Species</th>
<th>Forward Primer</th>
<th>AluI Restriction Site</th>
<th>Reverse Primer</th>
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</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Forward Primer</th>
<th>CViKI-1 Restriction Site</th>
<th>Reverse Primer</th>
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</table>

Figure 1. (colour online) In silico analysis of the monkey-specific primers: (a) dendrogram built from the 120-bp regions of d-loop gene sequences of monkey and other 17 land and aquatic species using the neighbour-joining method; (b) 3D plot for primer mismatch and pairwise distance; and (c) mismatch bases of studied species with monkey-specific primers along with AluI and CViKI-1 restriction sites.
resulted following \textit{Alu} digestion of the PCR product. However, an 11-bp fragment which was below the resolution capacity of the instrument (15 bp) could not be detected. Meanwhile, lane 7 of Figure 4 presents the \textit{CViKI-1} digestion product (three fragments of length 45, 31 and 20 bp). The other fragments (13 and 11 bp) were below the lower end resolution and, hence, remained undetected. On the other hand, endogenous control (141 bp) produced two \textit{Alu} (127 and 14 bp) and four \textit{CViKI-1} fragments (73, 39, 15 and 14 bp). However, only 127-bp (lane 4) and 73- and 39-bp (lane 7) fragments were detected.

We prepared two sets of mixed-meat products (monkey–beef and monkey–chevon) to simulate the most potential forms of adulteration to detect adulterated monkey meats in processed meats. The admixtures contained 100%, 10%, 5%, 1% and 0.1% of spiked monkey meat in a balanced amount of beef and chevon (Figure 5: only monkey–chevon data are presented for simplicity). Monkey-specific PCR product (120 bp) was obtained from all levels of adulteration, even as low as 0.1% (w/w) of spiked monkey meat in beef and chevon. An endogenous 141-bp eukaryotic target was amplified from all admixed, reflecting good-quality DNA in all admixtures, eliminating the chances of any false-negative detection. We further confirmed the monkey-specific PCR product amplified from mixture backgrounds by digesting them with \textit{Alu} and \textit{CViKI-1}. It has been reported that meat admixtures are not suitable for PCR-RFLP analysis since the digestion results might show a combination of miscellaneous restriction patterns for all possible species contained in the adulterated sample (Fajardo et al. 2007). However, we successfully amplified only the targeted products and its digestion products were similar to those from pure background (Figure 5).

**Commercial meatball analysis**

In the food industry, the replacement of costly meats by cheaper products is quite a common practice to increase profits. Therefore, we screened commercial meatball
samples using 0.1% monkey meat-spiked dummy meatballs as a positive control. The model meatballs were prepared following Ali, Hashim, Mustafa, Che Man, Dhahi, et al. (2012). In total eight different ‘halal’-branded chicken (A–D) and beef meatballs (E–H) were purchased from different Malaysian stores and then tested (Table 1).

While the monkey PCR product was obtained from all positive controls, no commercial meatballs collected from different outlets were found to be positive for monkey DNA (Figure 6), reflecting the absence of monkey meat adulteration in meatball formulations in Malaysia. Amplification of endogenous eukaryotic control reflected

Table 1. Analysis of monkey meat in commercial chicken and beef meatball products.

<table>
<thead>
<tr>
<th>Chicken meatball</th>
<th>Number of sample</th>
<th>Positive detection</th>
<th>Beef meatball</th>
<th>Number of sample</th>
<th>Positive detection</th>
<th>Replicate</th>
<th>PCR accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3</td>
<td>0/9</td>
<td>E</td>
<td>3</td>
<td>0/9</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>0/9</td>
<td>F</td>
<td>3</td>
<td>0/9</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>0/9</td>
<td>G</td>
<td>3</td>
<td>0/9</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>D</td>
<td>3</td>
<td>0/9</td>
<td>H</td>
<td>3</td>
<td>0/9</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>Positive control (0.1% monkey meat)</td>
<td>3</td>
<td>9/9</td>
<td>Positive control (0.1% monkey meat)</td>
<td>3</td>
<td>9/9</td>
<td>3</td>
<td>100</td>
</tr>
</tbody>
</table>

Note: The numerator and denominator of each fraction denote the number of positive detection and total number of samples analysed.

Figure 4. Restriction pattern analysis of monkey-target (120 bp) and endogenous control before (lanes 1–3) and after AluI (lanes 4–6) and CVIK-I digestion. Lanes 1, 4 and 7: monkey; lanes 2, 5 and 8: cow; and lanes 3, 6 and 9: goat.

Figure 5. Specificity and sensitivity analysis in binary mixture of monkey and chevon. Lane L: DNA ladder; lanes 1–4 and 7: 100%, 10%, 5%, 1% and 0.1% of monkey meat in a balanced amount of chevon. Restriction patterns of PCR product was obtained from 1% (lanes 5 and 6) and 0.1% (lanes 8 and 9) monkey in chevon after AluI and CVIK-I digestion. Lane 10: 100% chevon.
good-quality DNA in all commercial products. The findings are acceptable from a Malaysian perspective since the country is committed to develop a halal-hub industry and has been strictly monitoring the halal standard of marketed foods.

Conclusions

The growing extinction or threats of extinction of some wild species from the world map have made us think about the protection of game animals such as macaque monkeys that are widespread in tropical forests, but which continue to be threatened in many places by intentional killing for meat or the selling of meat on the black market. Wider availability, improved meat quality and lack of awareness might allure some people to bring monkey meats into the food chain under the banner of common meats such as beef, goat, chicken, duck, turkey and pork as well as their meat products. However, the threats to wild species are not limited here. Macaques might also be the potential carriers of some deadly diseases such as hepatitis, anthrax and simian immunodeficiency virus (SIV). Consumption of wild meat unbeknown to the consumer might lead to a sudden allergic reaction in certain individuals. Thus, regulatory bodies for foods and feeds along with wildlife protection agencies need a reliable authentication technique for the unambiguous tracing of monkey meats under various matrices including the processed foods.

For the first time we developed here a PCR-RFLP assay coupled with a micro-fluidic-chip-based automated electrophoresis system which is a promising tool to verify short-length PCR products or nucleic acid fragments. We unambiguously amplified a very short-length site (120 bp) of macaque’s d-loop gene which is present in multiple copies per cell and protected my mitochondrial membranes. This short-length target was found to be extremely stable under extensive boiling, autoclaving and microwaving treatments which are known to degrade longer DNA targets extensively, suggesting its application reliability even for compromised or extensively degraded specimens. Additionally, distinctive restriction patterns with enough macaque fingerprints were obtained upon AluI and CViK-1 digestion followed by automated separation both under pure and complex matrices, further confirming the authenticity of the amplified target. We used an endogenous control (141 bp) that eliminated the chances of any false-negatives. We optimised and adapted the assay to screen commercial meatball products which are very popular in Malaysia and most parts of the globe. The LOD of the assay was 0.00001 ng monkey DNA in a pure state and 0.1% (w/w) monkey meat in meat mixtures and meatball formulations. To the best of our knowledge, there are no published reports of such a reliable, sensitive and validated assay suitable for the tracking and tracing of macaque meats under pure, admixed and commercial matrices, even for highly degraded specimens. We strongly believe the assay would be valid for any forensic or archaeological identification of macaque species, even under compromised states.

Acknowledgements

The authors are thankful to the Department of Wildlife and National Park Malaysia for the donation of macaque meat tissue samples for conducting this study.

Disclosure statement

No potential conflict of interest was reported by the authors.
Funding
This research was supported by the University of Malaya Grand Challenge [grant number GC001-A-14SBS] to M. E. Ali.

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