DNA forensics in combating food frauds: a study from China in identifying canned meat labelled as deer origin

Mukesh Thakur[1,7,*], Ruheena Javed[2], Ved Prakash Kumar[3], Malay Shukla[4], Neha Singh[5], Aishwarya Maheshwari[3], Nipun Mohan[6], Dong-Dong Wu[1] and Ya-Ping Zhang[1]

[1]State Key Laboratory of Genetic Resources and Evolution and Yunnan Laboratory of Molecular Biology of Domestic Animals, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, Yunnan, People's Republic of China
[2]Guangzhou Institute of Pediatrics, Guangzhou Women and Children's Medical Center, Guangzhou 510623, Guangdong, People's Republic of China
[3]Wildlife Institute of India, Dehradun 248 001, India
[4]Gujarat Forensic Sciences University, Gandhinagar 382 007, India
[5]University School of Environment Management, Guru Gobind Singh Indraprastha University, Delhi 110 078, India
[6]Allahabad High Court, Allahabad 211 001, India
[7]Present address: Molecular Systematics Division, Zoological Survey of India, Kolkata 700 053, India

Now-a-days processed and canned food products are consumed in increasing quantity in all developing and developed countries following changes in human lifestyle. However, customers are often fooled by products they buy and such frauds frequently go unreported since species identification requires technical inputs and considerable time and efforts. We bought two canned meat packets labelled as 'classic delicious deer meat' from Shanghai Pudong International Airport, Shanghai (People's Republic of China) to understand its origin as deer meat is legally prohibited in many parts of the world. In this study, we screened these samples with DNA barcoding approach using conserved mitochondrial genes. Homology search on NCBI and phylogenetic analysis identified these samples to have originated from a domestic pig of China. We propose that the methodology used is appropriate for identifying the processed and canned food products and further suggest to check the labelling regulations to guarantee the protection of consumers' rights.

Keywords: Canned food, DNA forensics, food frauds, mitochondrial genes, species identification.

DELIBERATE falsification of food content on the product label is a common practice, particularly with high value-added products commanding a premium price since customers often go for brands and never feel the necessity to check the originality of food commodities. Barely, there is a check on the integrity and novelty of food products sold in shopping malls, grocery shops and duty-free stores in airport premises. It is consumer's right to set the exact information on the products they buy. Manufacturers must honestly describe the constituents used/packed in processed and canned food items, as the information is liable to influence consumers' decision whether to buy or not to buy the product considering his/her lifestyle or religious belief (e.g. vegans prefer organic products, many Hindus, Muslims and Jews do not prefer to eat pork) or even health issues (e.g. people often avoid certain type of meat that can induce particular allergies). Therefore, description and labelling of food must be truthful and accurate, particularly for processed and canned food items where no morphological identity is provided leading to the identification of meat. Further, adding flavours in such canned foods change the essence of meat to make it difficult to assign them to a specific meat type. Consuming certain type of meat or meat products is considered a taboo in some parts of the world while there are numerous instances where food substitution and adulteration have been reported. A recent study reported mislabelling of food products and documented about 20–70% substitution rate in the marketed meat products[1]. Selling certain type of meat and meat products is governed by various laws depending on the countries' faunal resources and status enacted by laws prevailing in such countries. Sometimes meat of a threatened species is also sold in the name of other species that is permissible by law thereby committing a serious wildlife crime by disguise, which becomes unsustainable when traded on a commercial scale. Despite the existing national and international food law regulations, it is impossible to control large scale malpractices from harming the consumers[2].

To prove fraud requires detection and identification of food constituents, which is often challenging and needs technical inputs from experts[3,4]. Mislabelling food products has been reported by different chemical and biochemical techniques. In recent years, DNA analysis has taken a central role in identifying frauds mislabelling[5–11]. Certain meat types are rich in protein content and are thought to be used as a remedy to cure malnutrition or other diseases. However, their specific uses are not well documented. Therefore, consumers all over the world often get attracted to buy such mislabelled or substituted products for medicinal values and get cheated. Sometimes they also suffer from various ailments since they unknowingly eat undesirable meat. Based on a general market survey, it can be interpreted that consumers are aware of food safety, but incomplete knowledge of safe food and recognition of mislabelled food, drive them to purchase what is not preferred. In the present scenario, where food crimes are rampant, it is necessary that consumer's rights are protected and modern techniques like DNA barcoding used as a handy tool to protect consumer rights and related laws.

While travelling within the People's Republic of China (PRC), one of the authors bought two packets of canned

*For correspondence. (e-mail: thamukesh@gmail.com)

meat product from a duty-free shop at Shanghai Pudong International Airport, Shanghai. These packets were printed with deer photos and labelled as 'classic delicious deer meat' (Figure 1). The printed photos on food packets resembled Sika deer (*Cervus nippon*) at first glance. In China, three subspecies of Sika deer inhabit (*C. n. hortulorum*, *C. n. sichuanicus* and *C. n. kopschi*). Sika deer was listed 'endangered' in 1996 and 2003 by IUCN, and listed 'endangered' in the China Red Data Book of Endangered Animals[12]. Its present conservation status under IUCN laws is of least concern which shows a recent expansion of Sika deer in numbers and distribution range[13], while it is still listed as a National Class I Protected Wild Animal Species of China. The purchased packets were not intended for consumption, but to verify the authenticity of actual species of origin of the canned meat products being sold in airport premises. Therefore, to satisfy our curiosity, we undertook this study to determine species of origin from these processed meat samples using DNA barcoding with conserved mitochondrial genes.

Both the canned meat packets were photographed and opened under controlled conditions. These samples were fully processed and seasoned with spices, herbs and condiments as they emitted a nice fragrance during handling and processing in the laboratory. We rinsed these samples thrice in 1× PBS (kept for at least 20 min on a shaking platform during each wash) and extracted genomic DNA using DNeasy Blood & Tissue Kit (Qiagen, Germany) following the manufacturer's instructions. We amplified partial fragments of three mitochondrial genes – cytochrome *b* (Cyt *b*), 16S rRNA and 12S rRNA with universal primers[14–16]. We carried out independent PCRs for amplification of these three mitochondrial genes on an Applied Biosystems thermal cycler (ABI, 2720). Each reaction of 10 µl reaction contained 1× PCR buffer (50 mM KCl, 10 mM tris–HCl), 2.5 mM of $MgCl_2$, 200 µM of each d-NTP (deoxy-nucleotide triphosphate), 1.25 µg BSA (Bovine serum albumin), 4 pM of each primer (forward and reverse) and 0.5U of *Taq* DNA polymerase (MBI, Fermentas) and approximately 15–20 ng of genomic DNA. The cycling conditions were set up as: initial denaturation at 94°C for 2 min, 35 cycles (94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min) with a final extension at 72°C for 10 min. On completion of PCRs, we electrophorized PCR products on 2% agarose gel and observed over transilluminator to detect the amplification.

The PCR products were cleaned using Exo-SAP treatment, the residual oligonucleotides removed and the d-NTPs freed before DNA sequencing. We set up cycle sequencing PCR independently with forward and reverse primers of all three genes using the big dye terminator cycle sequencing kit® v 3.1 (Applied Biosystems, Foster City, USA). Any unbound dd-NTPs were removed using alcoholic precipitation method and sequencing performed on ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA).

The quality of sequences was checked using Sequence Analysis v 5.2 software (Applied Biosystems, Foster City, USA) and sequences validated manually, nucleotide by nucleotide using Sequencher v 4.7 software (www.genecode.com). The sequences were compared with NCBI/GenBank (http://www.ncbi.nlm.nih.gov/) database using BLAST tool and the most homologous sequences retrieved from NCBI database. In addition, we also downloaded sequences of several other species like Sika deer (*Cervus nippon*), wild pig (*Sus scrofa cristatus*), domestic pig (*Sus scrofa domesticus*), domestic goat (*Capra hircus*), cattle (*Bos taurus*), domestic buffalo (*Bubalus bubalis*), domestic dog (*Canis lupus familaris*), spotted deer (*Axis axis*), hog deer (*Axis porcinus*), sambar deer (*Rusa unicolor*) and barking deer (*Manutius muntjak*) for comparison. We performed multiple sequence alignment (MSA) using CLUSTAL W as implemented in BioEdit v 7.0.9.0 software[17] and constructed phylogenic trees based on Tamura 3 parameter model and neighbour-joining (NJ) method and the most fit substitution model for all the aligned sequences of these three genes in Mega v 5.0 (ref. 18).

Both samples yielded reasonable quality of genomic DNA and high quality DNA sequences for all the three genes. The sequences generated from the questioned meat samples (Q-01 and Q-02) were submitted as independent entries in BLAST search for retrieving the most similar sequences using the default mega blast algorithm parameters. We compared these sequences against those species that were likely to be hunted/in wildlife trade or consumed locally (Table 1). We trimmed sequences to bring them to a similar length for use in further analysis (Cyt *b*-342 bp, 16S rRNA-527 bp and 12S rRNA-393 bp). We recorded 100% homology in BLAST analysis between the sequences under question with domestic pig sequences for all three genes. Surprisingly, there was no homology of the suspected meat samples with deer sequences in

Figure 1. Processed meat products purchased from the Pudong Shanghai Airport, China.

Table 1. NCBI/GenBank accession numbers used in homology search through BLAST and in phylogenetic analysis

Scientific name	Cyt *b*	16S rRNA	12S rRNA
Cervus nippon (Sika deer)	D32192	KJ870170	D34627
Sus scrofa (Domestic pig China)	KR049169	KP223728	KP223728
Sus scrofa cristatus (Wild pig India)	JN039028	KT316288	KT316284
Sus scrofa domesticus (Domestic pig India)	JN039030	KT316290	KT316286
Capra hircus (Domestic goat)	AB044307	KF908864	AJ490504
Bos taurus (Cattle)	JX472273	AB074967	AF492351
Bubalus bubalis (Domestic buffalo)	FJ467917	JX666612	GU936495
Canis lupus familiaris (Domestic dog)	KJ660982	KF799980	AB048589
Axis axis (Spotted deer)	KP172494	JN093062	KP318118
Axis porcinus (Hog deer)	KP142685	KJ870167	AY775785
Rusa unicolor (Sambar deer)	DQ832274	EU223368	GQ463697
Muntiacus muntjak (Barking deer)	EU285566	AF108038	AM778453

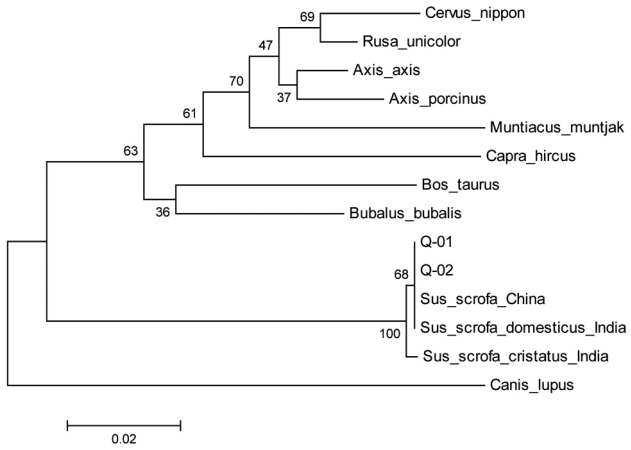

Figure 2. Neighbour-joining tree constructed based on aligned sequences of the partial fragment of mitochondrial Cyt *b* gene of different animals with Tamura 3 parameter model.

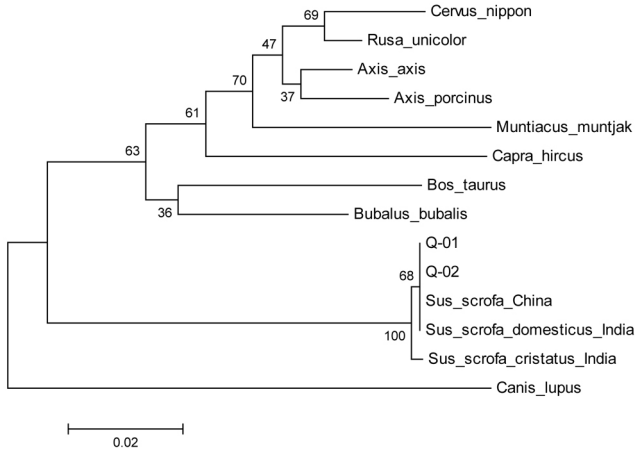

Figure 3. Neighbour-joining tree constructed based on aligned sequences of the partial fragment of mitochondrial 16S rRNA gene of different animals with Tamura 3 parameter model.

BLAST analysis indicating that these samples might have originated from a domestic pig of China. BLAST is an approximation to identify species to find regions of local

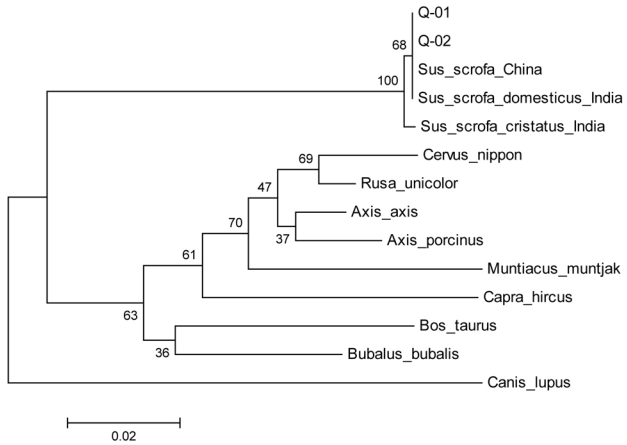

Figure 4. Neighbour-joining tree constructed based on aligned sequences of the partial fragment of mitochondrial 12S rRNA gene of different animals with Tamura 3 parameter model.

similarity between sequences. This method is similar to FINS (forensically informative nucleotide sequencing) technology, since it performs analysis based on DNA sequences and a database[19–22]. Further, the results were validated by phylogenetic analysis which grouped species with statistical bootstrap support. The assignments generated by the proposed BLAST were compared with results obtained by phylogenetic tree analysis. The NJ trees for all three genes also provided similar findings to that of BLAST and produced 100% bootstrap value that grouped questioned meat samples with Chinese domestic pig (Figures 2–4). The calculated bootstrap values higher or equal to 70% usually correspond to probabilities higher or equal to 95% which means that the topology is close to real[23], giving a quantitative measurement of certainty of the assignment of a sample to a particular species. The phylogenetic trees constructed using sequences of three mitochondrial genes of varying length (342 bp-Cyt *b*, 527 bp-16S rRNA and 393 bp-12S rRNA) showed that both the investigated meat samples belonged to individuals of the same species which was not different from a

domestic pig of China. All clusters were strongly supported, with bootstrap values of 100.

We have described here the utility of DNA barcoding using conserved mitochondrial genes in identifying species even from fully processed meat samples which might have undergone many processing and packaging steps before they were analysed. The study showed the importance of genetic analysis in wildlife forensics to curb frauds which often falsify or mislabel information on food products. Our investigation on canned meat samples which were labelled as deer meat actually turned out to be domestic pig meat. This study will help to have a legal check on the processed and canned food products being sold in shopping malls, grocery shops and in duty-free stores at airports premises at national and international levels. An important point to understand from the case study is that if consumer's confidence on food products is undermined, it gradually results in loss of faith on questioned products over a period of time. Consumers' rights protection laws should be strictly implemented and random scrutiny is inevitable from time to time to keep a check on such food frauds and mislabelling of food items.

1. Kane, D. E. and Hellberg, R. S., Identification of species in ground meat products sold on the US commercial market using DNA-based methods. *Food Control*, 2015, **59**, 158–163.
2. Ballin, N. Z., Authentication of meat and meat products. *Meat Sci.*, 2010, **86**(3), 577–587.
3. Woolfe, M. and Primrose, S., Food forensics: using DNA technology to combat misdescription and fraud. *Trends Biotechnol.*, 2004, **22**(5), 222–226.
4. Teletchea, F., Maudet, C. and Hänni, C., Food and forensic molecular identification: update and challenges. *Trends Biotechnol.*, 2005, **23**, 359–366.
5. Meyer, R., Höfelein, C., Lüthy, J. and Candrian, U., Polymerase chain reaction–restriction fragment length polymorphism analysis: a simple method for species identification in food. *J. AOAC Int.*, 1995, **78**, 1542–1551.
6. Unseld, M., Beyermann, B., Brandt, P. and Hiesel, R., Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. *PCR Methods Appl.*, 1995, **4**, 241–243.
7. Matsunaga, T., Chikuni, K., Tanabe, R., Muroya, S., Shibata, K., Yamada, J. and Shinmura, Y., A quick and simple method for the identification of meat species and meat products by PCR assay. *Meat Sci.*, 1999, **51**, 143–148.
8. Arahishi, F., PCR-RFLP analysis of nuclear nontranscribed spacer for mackerel species identification. *J. Agric. Food Chem.*, 2005, **53**, 508–511.
9. Wong, E. and Hanner, R., DNA barcoding detects market substitution in North American seafood. *Food Res. Int.*, 2008, **41**, 828–837.
10. Hellberg, R. S. and Morrissey, M. T., Advances in DNA-based techniques for the detection of seafood species substitution on the commercial market. *J. Lab. Autom.*, 2011, **16**, 308–321.
11. Shokralla, S., Hellberg, R. S., Handy, S. M., King, I. and Hajibabaei, M. A., DNA mini-barcoding system for authentication of processed fish products. *Sci. Rep.*, 2015, **5**, 15894; http://doi.org/10.1038/srep15894.
12. McCullough, D. L., Jiang, Z. G. and Li, C. W. (eds), In *Sika Deer: Biology and Management of Native and Introduced Populations*, Springer, Tokyo, New York, 2009, pp. 521–539; doi:10.1007/978-4-431-09429-6_35.
13. Harris, R. B., *Cervus nippon*. The IUCN Red List of Threatened Species, Version 2015.2, 2015; www.iucnredlist.org (accessed on 28 August 2015).
14. Verma, S. K. and Singh, L., Novel universal primers establish identify of an enormous number of animal species for forensic application. *Mol. Ecol. Notes*, 2003, **3**, 28–31.
15. Mitchell, S. E., Cockburn, A. F. and Seawright, J. A., The mitochondrial genome of *Anopheles quadrimaculatus* species A: complete nucleotide and organization. *Genome*, 1993, **36**, 1058–1073.
16. Kocher, T. D. *et al.*, Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 6196–6200.
17. Hall, T. A., BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symp. Ser.*, 1999, **41**, 95–98.
18. Tamura, K. *et al.*, MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.*, 2011, **28**, 2731–2739.
19. Bartlett, S. E. and Davidson, W. S., FINS (forensically informative nucleotide sequencing): a procedure for identifying the animal origin of biological specimens. *Biotechniques*, 1992, **12**, 408–411.
20. Guha, S. and Kashyap, V. K., Molecular identification of lizard by RAPD and FINS of mitochondrial 16s rRNA gene. *Legal Med. Tokyo*, 2006, **8**, 5–10.
21. Sahajpal, V. and Goyal, S. P., Identification of a forensic case using microscopy and forensically informative nucleotide sequencing (FINS): A case study of small Indian civet (*Viverricula indica*). *Sci. Justice*, 2010, **50**, 94–97.
22. Mukesh, Singh, S. K., Shukla, M., Sharma, L. K., Mohan, N., Goyal, S. P. and Sathyakumar, S., Identification of Galliformes through forensically informative nucleotide sequencing (FINS) and its implication in wildlife forensics. *J. Forensic Res.*, 2013, **4**, 195; doi:10.4172/2157-7145.1000195.
23. Hillis, D. M. and Bull, J. J., An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst. Biol.*, 1993, **42**, 182–192.

ACKNOWLEDGEMENTS. M.T. is grateful to the CAS-President's Fellowship (PIFI) to provide him financial support during his stay at Kunming Institute of Zoology, Kunming, China.

Received 10 April 2016; revised accepted 5 January 2017

doi: 10.18520/cs/v112/i12/2449-2452