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Development of Molecular Tools to Differentiate Sri Lankan Wild Boar (*Sus scrofa affinis*) Meat from Exotic and Village Pig (*Sus scrofa domestica*) Meat

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ABSTRACT. *Reliable labelling of meat products is important in the food industry in order to ensure food safety, prevent fraud adulterations and avoid conflict with socio-religious practices. In Sri Lanka, wild boar meat is known to be substituted with pork to meet the demand. There are many court cases on illegal selling and transportation of wild boar meat. Thus, reliable methods to identify wild boar meat are necessitated. Therefore, the objective of the present study was to differentiate meat of wild boar from exotic and domestic swine using mitochondrial DNA markers. The entire mitochondrial DNA D-loop region was amplified using the forward primer, 5'CCAAGACTCAAGGAAGGAGA3' and reverse primer, 5'GGCGCGGATACTTGCATGTG3'. From the sequence analysis several repetitive sequences of 5'CGTGCGTACA (10 bp) was observed indicating sequence heteroplasmy in porcine mitochondrial DNA D-loop region. Avoiding these repetitive sequences D-loop exhibited 17 polymorphic sites which enable differentiation of Sri Lankan wild boar from exotic and village pigs. Furthermore, a unique repeat of AAACCACAC (9 bp) was observed within the Sri Lankan wild boar samples analyzed. Targeting two polymorphic sites PCR-RFLP analysis was performed, using forward primer 5'GTGCTACGAAAGCAGG3' and same reverse primer followed by a simple restriction digestion using cost effective Dra 1 enzyme. Upon restriction digestion, wild boar produced two bands of 150 bp and 60 bp differing them from exotic and village pigs. This technique can be routinely applied to verify wild boar meat.*

Keywords: *Meat identification, mitochondrial DNA D-loop region, PCR-RFLP, wild boar*

INTRODUCTION

The increasing world population and the increasing demand for meat and meat products lower the per capita availability of protein of animal origin, resulting fraudulent substitution or adulteration of costly or highly demanded meat with cheaper ones (Koh *et al.,* 1998). Awareness on fraud substitutions or adulterations is necessary in the food industry, considering the food habits of the individuals. For example, some may have food allergies to certain meat species: Jews and Muslims consume only specified food types approved by their religion such as 'Kosher' and 'Halal' respectively. Hindus do not consume beef due to

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religious dietary restrictions and vegetarians totally restrict the consumption of meat. Thus, the knowledge on exact source or origin of animal protein is crucial in food safety where the consumers demand the traceability of meat from farm to fork.

Despite the high price, game meat is highly popular and highly demanded in meat industry, where substitutions and adulterations are commonly seen. Game meat is favored by many people due to diverse reasons such as intense flavor, specific texture, low fat and cholesterol content, lack of anabolic steroids and other drugs and increased awareness on organic products (La Neve *et al*., 2008; Hoffman & Wiklund, 2006).

Though, game animal hunting is popular in many countries, hunting of wild animals is restricted by law in Sri Lanka. Among the game meats in the country, wild boar meat is highly popular. Although, the conservation status of wild boar is not classified as threatened or near threatened, hunting of wild boar is restricted in the country. The Fauna and Flora Ordinance in Sri Lanka states; "*no animal shall be hunted, killed or taken and no plant shall be damaged, collected, or destroyed, in a strict natural reserve, national park, nature reserve or jungle corridor*" (3,44 of 1964). The ordinance also bans the sale or transport of wild boar meat, even that of wild boars killed under the exemption for animals that damage crops. There are abundant court cases due to illegal selling and keeping of wild boar meat. When wild animals are hunted, phenotype markers are often destroyed or intentionally removed to conceal the species of origin, misleading the relevant authorities of wild life conservation (Rastogi *et al.*, 2007). Therefore, identification of wild boar meat is important in making correct and fair decision in keeping with the country's law and order.

On the other hand, wild animals are valuable genetic resources which can be effectively utilized for sustainable food supply in the future. Therefore, to safeguard the wild animals as well as to avoid economic fraudulence, it is required to have reliable and sensitive methods of meat identification (Dooley *et al*., 2004). Sensory analysis, anatomical differences, histological differentiation of the hair that may exist on the meat, properties of tissue fat, level of glycogen in muscle tissue and protein based techniques (electrophoretic, chromatographic, immunological assays) are some of the methods used for meat identification (Ashoor *et al.*, 1998; Arslan *et al.*, 2005). But due to problems of specificity, some of these methods are inadequate for routine analysis (Arslan *et al.*, 2005).

DNA-based techniques are now widely used to identify the species of origin of meat due to its ubiquitous nature (Wolf *et al*., 1999). DNA is a stable molecule under high temperature, high pressure and chemical treatments used in processing (Arslan *et al.*, 2006). DNA-based methods for meat identification include Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) (Fajardo *et al*., 2006), Random Amplified Polymorphic DNA (RAPD), DNA hybridization and Single Strand Conformational Polymorphism (SSCP) (Dooley *et al*., 2004).

Both nuclear and mitochondrial DNA (mtDNA) have been used for these studies. In the case of mtDNA, cytochrome *b* gene (Hwang *et al*., 2004), 12S (Fajardo *et al*., 2008), 16S (Rastogi *et al*., 2007), 18S (Meyer *et al*., 1995) ribosomal RNA (rRNA) subunits and displacement loop region (D-loop) (Fajardo *et al*., 2007) have been used for species identification. Due to maternal inheritance of mtDNA no recombination mechanism exists as in the nuclear DNA to eliminate error once a mutation occurred (Hwang *et al*., 2004). Thus, accumulation of these point mutations allows discrimination of closely related species (Wolf

et al., 1999). However, attempts on within species identification using these methods are scanty.
Therefore, identification of meat within species, at least at subspecies or breed levels need to be further investigated. Thus, objective of the present study is to identify the meat of wild boar from exotic swine breeds and village type using mtDNA based techniques.

MATERIALS AND METHODS

The experiments were carried out at the Agriculture Biotechnology Centre, Faculty of Agriculture, University of Peradeniya, Sri Lanka and CAAS-ILRI Joint Laboratory on Livestock and Forage Genetic Resources (JLLFGR), Institute of Animal Science, Chinese Academy of Agricultural Sciences (CAAS), Beijing, China.

Sample collection and storage

Meat or blood samples of wild boar (*Sus scrofa affinis*) were obtained from Uda Peradeniya Livestock Experimental Field Station, University of Peradeniya and from Gampola, Galaha, Kadugannawa and Kegalle areas. Local domestic pig samples were collected from Negumbo and Beruwala coastal areas along the western side of the island where the local pigs were reared extensively. The commercial pig samples of 'Large white', 'Land race' and 'Duroc' breeds were collected from a commercial private farm (Chutiduwa farm, Katuneriya, Negumbo) and from Uda Peradeniya Livestock Experimental Field Station, University of Peradeniya. Meat and blood samples were stored at -20°C and 4°C respectively until they were used for the analysis.

DNA extraction

Genomic DNA was obtained from meat using the salting-out protocol as described by Jianlin (2004).

DNA quantification

DNA quantification was carried out using NanoDrop spectrophotometer ND1000 (Thermo Fisher Scientific, Wilmington, DE, USA). The measurement (ng/µl) was obtained using the PC based software.

DNA amplification

PCR amplification of mtDNA was done at three different as follows.

Amplification of a fragment of mtDNA D-loop region

A fragment of the mtDNA D-loop region was amplified as described by Montiel-sosa *et al*. (2000). The following primer pair was used for the amplification.

pig F 5' AACCCTATGTACGTCGTGCAT (15592)	primer 1F
pig R 5' ACCATTGACTGAATAGCACCT (16124)	primer 1R

Amplification of DNA was carried out in a final volume of 50 µl in tubes containing total DNA (20 ng/µl), 10 x reaction buffer, 10 mM of each dATP, dCTP, dGTP, dTTP (Promega, Madison, WI , USA), 20 pmol/µl of each primer (Genetech, Sri Lanka) and 5 U of *Taq* DNA polymerase (Promega, Madison, WI , USA). After 5 min of initial denaturation at 94 °C, 30

cycles of amplification at 94 °C for 45 sec (denaturation), 62 °C for 45 sec (annealing), and 72 °C for 1 min (elongation) were carried out in a thermal cycer (Applied biosystems, Foster city, California). Amplified products were electrophoresed in a 2 % agarose gel in TBE buffer, observed by staining with ethidium bromide under UV transillumination (BINTA 2020D).

Amplification of the entire mtDNA D-loop region

Complete D-loop mtDNA was amplified using the primer pair given below.

L-strand (forward) (5'CCAAGACTCAAGGAAGGAGA3')	primer 2F
H-strand (reverse) (5'GGCGCGGATACTTGCATGTG3')	primer 2R

PCR amplification was performed in a gene Amp PCR systems 9700 (Applied biosystems, Foster city, CA, USA). The PCR reaction was performed in a total volume of 50 µl, containing 50 ng/µl of genomic DNA, 10 ng/µl of both forward and reverse primers, 2.5 mM of dNTP, 10 x reaction buffer, 5 U *taq* polymerase (Tiangen, Beijing, China). The 30 cycles of amplification was carried out with 94 °C 30 sec (denaturation), 60 °C 45 sec (annealing), and 72 °C 90 sec (elongation). The cycles were initiated by initial denaturation at 94 °C for 5 min and terminated by final extension at 72 °C for 10 min. Amplified products were electrophoresed in a 2 % agarose gel in TBE buffer, observed by staining with ethidium bromide under UV transillumination (BINTA 2020D).

The amplified products were sequenced (Beijing Sunbiotech, China) and the sequences obtained were aligned using the Chromas software package version 2.0 and Mega software packages version 5.0.

PCR-RFLP analysis of mitochondrial D-loop region

Restriction maps were developed for wild boar, local and domestic pigs. The sequences were analyzed by Cleaver and Primer premier 5 software packages. After detailed comparison a cost effective enzyme *Dra I* was selected.

The following new forward primer (3F) was designed to amplify only the target polymorphic site of mtDNA D-loop region along with the 2R reverse primer.

5'GTGCTACGAAAGCAGG3'	primer 3F

RESULTS AND DISCUSSION

Mitochondrial DNAs are used extensively for meat identification studies due to its higher evolution rate and sequence diversity compared to nuclear DNA (Wolf *et al*., 1999). On the other hand, mtDNA is maternally inherited and only one allele exists in an individual. Therefore, accumulated point mutations can be easily detected in the sequence analysis due to absence of recombination mechanisms facilitating discrimination of closely related species (Hwang *et al*., 2004). Furthermore, only a small amount of the sample will be adequate for mtDNA analysis due to presence of a large number of mitochondria in each cell (Montiel-sosa *et al*., 2000). The D-loop is included in the control region of the mtDNA and is flanked by the tRA^{pro} and tRA^{phe} mt genes (Sbisa *et al*., 1997). MtDNA D-loop region has the highest

substitution rate and is the most rapidly evolving region of the mitochondrial genome. Thus, mtDNA D-loop region was targeted in this study for identification of wild boar meat from exotic and village pigs.

Polymorphisms in the mitochondrial DNA D-loop region

Amplifiaction of DNA by the primers 1F and 1R resulted in amplicons of ~500 bp (data not shown). Single nucleotide point mutations generated from these partial D-loop sequences were not adequate to differentiate wild boar from other pigs. However, Montiel-sosa *et al*. (2000) distinguished wild boar from pork using a restriction site derived from one base deletion in wild boar with respect to pig mitochondrial DNA. But this deletion was not observed in this study. Therefore, amplification of entire D-loop was carried out to explore single nucleotide point mutations.

The primer pair 2F and 2R described above amplified a region of ~1200 bp from swine but failed to produce any detectable amplicons from chicken, sheep and yak (Fig. 1). Thus, the primer pair used is sensitive enough to specifically amplify the swine D-loop region.

The amplified nucleotide sequence of swine includes several repetitive sequences of 5'CGTGCGTACA (10 bp) in the D-loop indicating sequence heteroplasmy. Though, *Sus scrofa* mitochondrial genome is about 16 kb, the length is not specific due to presence of these tandem repeats (5' CGTGCGTACA) in the D-loop (Lin *et al*., 1999). According to Ghivizzani *et al*. (1993) this 10 bp sequence is repeated tandemly 14-29 times in different porcine mitochondrial genomes. The intracellular variability may be due to the repeated and self complementary properties of this sequence which would favour mispairing and lead to replication slippage.

Thus, forward and reverse compliments of 17 wild boars, 20 village pigs and 27 exotic pig samples collected from Sri Lanka were aligned separately as forward complement and reverse complement avoiding the tandem repeats to find out intra-species nucleotide polymorphisms.

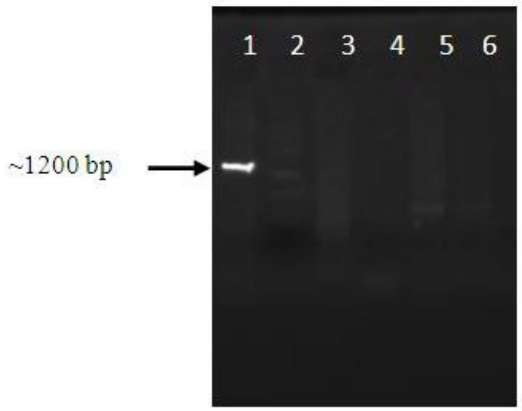

Fig. 1. Electrophoretic analysis of the PCR products amplified from wild boar (lane 1), chicken (lane 3), sheep 1 (lane 4), shep 2 (lane 5), yak (lane 6). Molecular weight marker III, 200 – 4500 bp (lane 2)

The aligned sequences were compared with sequence data from Larson *et al*. (2010) (from 16 countries representing Asia and Pacific) and sequences downloaded from National Centre

for Biotechnology Information (NCBI) data base to find a polymorphic DNA region. After detailed comparison, 8 and 9 polymorphic sites in wild boar were identified from forward and reverse complements (Table 1), respectively. Apart from these polymorphisms, a repeat of AAA CCA CAC (Fig. 2) (9 bp) was observed within the reverse complement in all the wild boars except WB 7, 8 and other downloaded sequences. Despite the two negative wild boar samples it was confirmed that this insertion is unique to Sri Lankan wild boar.

Table 1. Summary of polymorphic sites which can be used to differentiate Sri Lankan wild boar from village pigs and exotic pigs (Duroc, Land race and Large white)

	Polymorphic site no.	Position[a]	Sri Lankan wild boar (WB 1 - 6 & 9 -17)[b]	Sri Lankan wild boar (WB 7 & 8)	Others*
Forward	1	15,452	C	T	T
	2	15,523	C	G	G
	3	15,562	T	C	C
	4	15,756	G	A	A
	5	15,841	A	T	T
	6	16,097	T	C	C
	7	16,103	G	A	A
	8	16,108	T	C	C
Reverse	1	16,674	G	A	A
	2	16,682	C	T	T
	3	16,690	T	C	C
	4	16,703	T	A	A
	5	16,742	C	T	T
	6	16,774	T	C	C
	7	16,887	T	C	C
	8	16,925	T	C	C
	9	16,927	A	G	G

[a]Position of the nucleotide substitution according to FJ237003 (Alves *et al*., 2009)
[b] Gene bank accession numbers of wild boar samples (reverse only): WB 1-6: JN858911-JN858916; WB 9-17:JN858917-JN858925.
*Village pig (VP1-20), Duroc (D1-12), Land race (LR1-8), Large white (LW1-7)

Interestingly, the two wild boar samples (WB 7 & WB 8) used in the study were different from all other wild boars. The preliminary mtDNA analysis revealed that those two samples were homologous to village and exotic pig sequences. The most probable reason for this sequence diversity is crossing of wild boars to exotic sows. Though, wild boar meat is favored by many due to its intense flavor compared to exotic pig meat, wild boar meat is less available in the island due to reasons such as laws enforced. Thus, crossing of exotic sows to wild boar and selling of meat from crossbred offspring as wild boar meat is a common practice in the country (Chandrasiri, 2004). Therefore, to facilitate mating with wild boar the exotic sows were known to be tied up in the jungles (Subalini *et al*., 2010).

Though, the village pigs were assumed to have evolved as a result of gradual domestication of wild pigs of Sri Lanka (Rajamahendran *et al*., 1986), the village pig sequences obtained in this study suggests that they resemble more of exotic breeds rather than wild pigs. According to Chandrasiri (2004) there were recent introgression of exotic genome due to the state sponsored programs conducted to upgrade the local population with imported semen or live

animals of Large white, Land race and Duroc breeds. In most of these upgrading programs paternal and not maternal introgression is possible as it is the imported semen or boar that is used commonly in the breeding program. Since mt DNA is always maternally originated, according to the present findings the origin of domestic local pig becomes questionable. However, the polymorphism observed in D-loop sequence can be used to differentiate Sri Lankan wild boar from village and exotic pigs from Sri Lanka and from other Asian Pigs even though they are closely related.

Restriction fragment length polymorphism (RFLP) analysis of D-loop region

PCR-RFLP is one of the main genetic tools adopted by researchers to distinguish meat species. Thus, PCR-RFLP technique was a tool of choice in this study in order to differentiate meat of wild boar from others owing to the advantages such as simplicity, low cost, speed and better resolving power compared to DNA sequencing and sequence analysis (Fajardo *et al*., 2006).

In order to facilitate better detection of restriction fragments, a new forward primer (3F) along with the reverse primer, 2R was used to amplify a DNA fragment (~270 bp) targeting the restriction site which was derived using the 8th and 9th polymorphic sites of the reverse complement (Table 1 & Fig. 2). The cleavage pattern obtained after digestion with *Dra I* restriction enzyme is given in Fig. 3. The amplified DNA fragment of wild boar produced two bands of 150 bp and 60 bp upon restriction digestion where as exotic and village pig samples produced two bands of 210 bp and 60 bp. Thus, use of the PCR-RFLP technique targeting the mtDNA D-loop region is a promising method to distinguish wild boar from exotic and domestic swine meats.

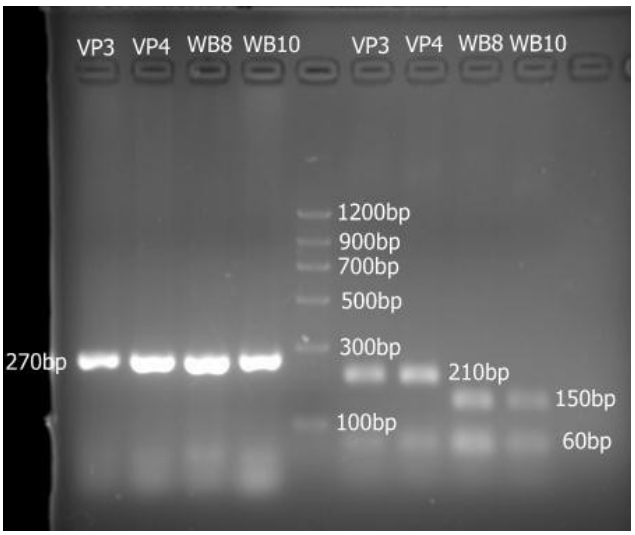

Fig. 3. PCR-RFLP patterns obtained after digestion of mt DNA amplicons with *Dra I* restriction enzyme (right) and undigested DNA 0f ~270 bp (left). Samples are, village pig (VP), wild boar (WB) & molecular weight marker (M)

However, chance of an intra-species mutation occurring at a restriction site and difficulties in detecting the crossbred individuals due to their maternal inheritance limits the use of mtDNA

in meat authentication. Hence, mtDNA data should be used together with alternative nuclear markers such as Melanocortin Receptor 1 gene (MC1R) (Fajardo *et al*., 2008), actin–intron region (Rastogi *et al*., 2007) and glucosephosphate isomerase-processed pseudogene (GPIP) (Naya *et al*., 2003). The second phase of this study deals with use of nuclear markers for meat identification.

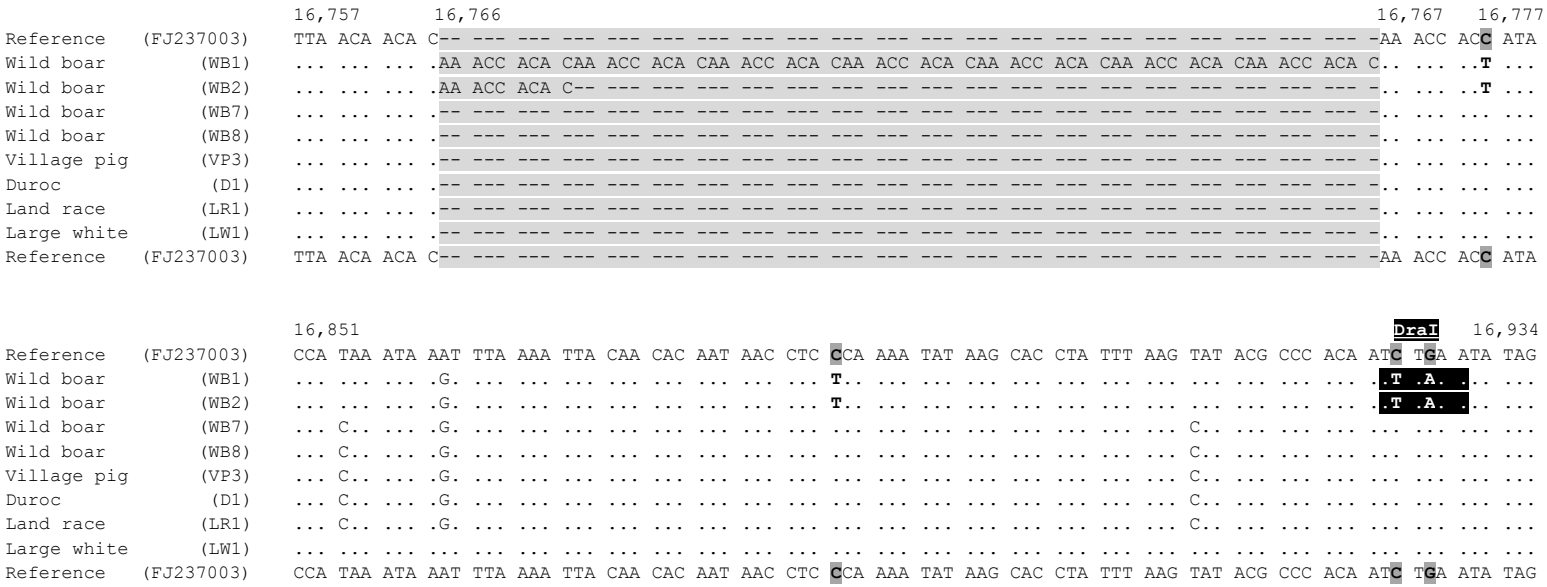

Fig. 2. DNA sequence alignment of part of the mitochondrial D-loop region of selected wild boar, village pig and exotic pigs (Duroc, Land race & Large white) from Table 1. Dot (.) and dash (-) indicates, nucleotide identity and deletions respectively, according to the reference sequence (FJ237003). Bold and highlighted nucleotide positions in the reference sequence indicate the polymorphic sites of wild boar from other pigs. The region used for restriction analysis (DraI - TTTAAA) is shown with shadow. The highlighted sequences indicate the AAACCACAC repetitive sequence in Sri Lankan wild boar. Numbering is according to the reference sequence excluding deletions.

CONCLUSIONS

Specific amplification of mtDNA D-loop region, followed by restriction analysis of amplicons with *DraI* endonuclease, provide a valuable basis for differentiating wild boar from domestic swine, offering a useful tool to reveal fraud in meat substitutions as well as in legal cases to verify wild boar meat.

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