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RESEARCH ARTICLE
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Development of molecular tools to differentiate Indian wild pig (*Sus scrofa cristatus*) meat from exotic and local domestic pig meat

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Abstract

Aim: Identification of wild pig and domestic pig is essential to prevent illegal poaching of wild pig and to implement Wildlife (Protection) Act, 1972. PCR-RFLP was used to differentiate Wild pig (*Sus scrofa cristatus*) from Domestic pig (*Sus scrofa domestica*) meat.

Materials and Methods: DNA was isolated from meat samples of both the sub species and a fragment of Cytochrome b gene was amplified using universal primers and the PCR products were subjected to restriction digestion.

Results: All the known samples of each of the sub-species amplified 474 bp fragment successfully using b1 and b2 primers. To differentiate between wild and domestic pig meat, restriction digestion of the PCR products was carried out to produce characteristic PCR-RFLP patterns for each species. *Stu*I digestion yielded a RFLP pattern which distinguished the closely related sub species. The alignment of sequences of Wild pigs with sequences of local domestic pig, European wild pig and exotic breeds revealed 7 intra-species polymorphic sites within Cytochrome b gene fragment.

Conclusion: This study showed that The PCR-RFLP is a simple and very effective tool for differentiating the samples of both the sub species and could prove to be a useful tool in forensic identification of wild pig and domestic pig.

Keywords: cytochrome b gene, domestic pig, Indian wild pig, PCR-RFLP

Introduction

Indian wild pig (*Sus scrofa cirstatus*) is a protected species under Schedule III of the Indian Wildlife (Protection) Act, 1972 and a separate sub-species from the domestic pig (*Sus scrofa domesticus*), which is a source of meat and an important farm animal in many states of India [1]. Indian wild pig is extensively poached for meat and in the absence of simple differentiation protocol for meat identification of wild and domestic pigs, implementation of Wildlife (Protection) Act, 1972 remains a constraint.

Most of the methods used for identification of species of origin of meat have been reported to have limitations in use due to problems in specificity (i.e. sensory analysis, glycogen level, histological technique, tissue fat properties & immunological methods), complexity (i.e. electrophoresis and DNA hybridization), high cost (i.e. DNA hybridization) and requirements of base line data (i.e. isoelectric focusing) [2]. DNA-based methods for meat identification include Polymerase Chain Reaction [3], Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) [4,5], Random Amplified Polymorphic DNA (RAPD) [6], Single Strand Conformational Polymorphism (SSCP) [7], nucleotide sequencing [8] and real time PCR [9]. Both nuclear and mitochondrial DNA (mtDNA) has been used for these studies [3-9]. Due to maternal inheritance of mtDNA, no recombination mechanism exists as in the nuclear DNA to eliminate error once a mutation occurred [10]. Thus, accumulation of these point mutations allows discrimination of closely related species [11]. However, attempts on within species or closely related sub-species identification using simple PCR-RFLP method are scanty.

The cytochrome b gene for species identifications have been used by many researchers as it is one of the bettered genes in the genbank and has superior ability for separating species when compared to other genes [12]. In the present study, the partial sequence of the Cytochrome b gene of Indian wild pig, domestic pig, European wild pig and exotic breeds were compared to develop a simple Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assay.

Materials and Methods

Five known samples (meat) of each domestic and wild pig collected from fresh carcass were used for DNA isolation using DNeasy Blood & Tissue Kit (QIAGEN, Germany) in a final elution volume of 300 µl. The DNA was subjected to PCR amplification in a Gradient Thermal Cycler (Eppendrof, India) in a final volume of 50 µl containing 200 ng of extracted DNA, 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 200µM of each dNTPs (dATP, dCTP, dGTP and dTTP), 1.5 units of Taq DNA Polymerase, 0.5µg/µl Bovine

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Table-1. Summary of polymorphic sites which can be used to differentiate Indian wild boar from local domestic pigs and exotic pigs (Duroc and Hampshire)

Polymorphic site no.	Position[a]	Wild pig	European wild pig/Exotic pig/local domestic pig
1	14310	A	G
2	14388	T	C
3	14395	A	G
4	14448	T	C
5	14466	C	T
6	14508	C	T
7	14517	T	C

[a] Position of nucleotide in reference to FJ237003

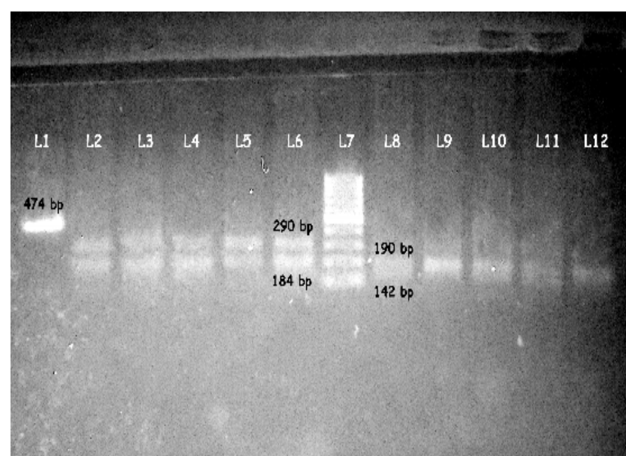

Figure-1. PCR-RFLP pattern produced by restriction digestion with *Stu*I (L1: Undigested PCR product, L2-L6: Domestic pig, L7: 100bp Ladder, L8-L12 : Wild pig)

Serum Albumin (BSA) and 20pmol of each primer. The universal primers of Cytochrome b gene fragment (b1-5' CCAATGATATGAAAAACCATCGTT 3' and b2- 5' GCCCCTCAGAATGATATTTGTCCTC 3') were used for PCR amplification [13]. The amplified product was subjected to electrophoresis on 1.2% agarose gel for 45 min at 70V in Tris Acetate EDTA (TAE) buffer and gel was stained with ethidium bromide (Et br) (0.5µg/ml). The amplification parameters were 95°C for 20 minutes followed by 35 cycles of 95°c for 1 min, 50°c for 1 min and 72°c for 1min with final extension at 72°C for 10 minutes prior to a 4ºC hold.

One PCR product of each subspecies were purified and sequenced in both directions using the BigDye® sequencing kit (Applied Biosystems, Foster City, California, USA). The obtained sequences have been submitted in Gene Bank under Accession No. KF185090 and KF185091. Three additional sequences of pig samples collected from forests of Western Ghats, one sequence each of European Wild pig, Hampshire and Duroc breed were retrieved from National Centre for Biotechnology Information (NCBI). The sequences were aligned using DNAstar program (DNAS Inc, Madison, WI, USA).

For differentiation, Cytochrome b gene fragment sequences were analyzed by DNAStar software and *Stu*I restriction enzyme (AGG↓ CCT) was used for RFLP analysis. The amplified PCR products were restriction digested with *Stu*I [14] in 30µl volume containing 3 µl 10X buffer, 10µl of amplicon, 1µl engyme, 17µl nuclease free water and incubated at 37ºC for overnight. The digested products were subjected to electrophoresis on 1.5% agarose gel at 1.5 V/cm for 4 hours and stained with Et br. Band patterns were analyzed by Gel documentation System (AlphaDigi Doc RT, JH India Ltd).

Results and Discussion

Due to the relatively high copy number of mtDNA genomes per cell, mtDNA is more suitable than nuclear genome for studies on degraded or otherwise compromised materials [15]. Various genes of mtDNA i.e. cytochrome *b* gene [16], 12S [17], 16S [18], 18S [19] ribosomal RNA (rRNA) subunit genes and displacement loop region (D-loop) [20] have been used for species identification.

All the known samples of each of the sub-species amplified 474 bp fragment successfully using b1 and b2 primers. Jon et al. [21] have also used this primer in the ARMs PCR to determine the presence of tiger bone in Traditional Chinese Medicine (TCM). We validated the sequence authenticity by direct sequencing of one PCR product each of Indian Wild pig and domestic local pig. Together with these sequences, a total of 8 mitochondrial sequences of Cytochrome b gene fragments were aligned to discern intra-species variations. Seven polymorphic sites were identified in the sequence of Indian wild pigs and sequences of samples collected from Western Ghats. Based on the intra-species variations, the Indian wild pig were discriminated by restriction digestion with *StuI* which generated bands of less than 200 bp (two 142 bp and one 190 bp bands) while two bands of 290 bp and 184 bp were observed for Indian wild pig and domestic pig, respectively, on 1.5% Agarose gel (Figure-1). Due to overlapping of bands of 142 bp only two bands (142 bp and 190 bp) are visible on restriction digestion with *Stu*I in wild pig. The RFLP patterns of *StuI* on amplified PCR product were highly specific. The sequence analysis of all the known samples of Indian wild pig revealed two *StuI* sites, while only one site of restriction was present in the amplified sequence of domestic pig, European wild pig and exotic breeds (Plate-1). The presence of 5th polymorphic site ("C") at the position of 14466 (Table-1) created an additional restriction site for *StuI*.

Apart from these polymorphisms, at the position of 14370 and 14407, all the sequences of Indian origin had "C" instead of "T" in European wild pig, Duroc and Hampshire pig breeds.

Srilankan wild boar (*Sus scrofa affinis*) was differentiated from native village pig (*Sus scrofa domestica*) targeting two polymorphic sites of mitochondrial D loop and restriction digestion with

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Reference(FJ237003)14297   TCCTAACAGGCCTGTTCTTAGCAATACATTACACATCAGACACAACAACAGCTTTCTCAT   14356
KF185091             182   .............A..............................................   241
Kf185090             182   ...................................C........................   241
FJ190153             153   .............A..............................................   212
FJ190159             154   .............A........................A.....................   213
Fj190161             153   .............A..............................................   212
AF486858             181   ............................................................   240
AY574046             181   ............................................................   240

Reference(FJ237003)14357   CAGTTACACACATTTGTCGAGACGTAAATTACGGATGAGTTATTCGCTATCTACATGCAA   14416
KF185091             242   .............C.................T......A..........C..........   301
KF185090             242   .............C...................................C...........  301
FJ190153             213   .............C.................T......A..........C..........   272
FJ190159             214   .............C.................T......A..........C..........   273
FJ190161             213   .............C.................T......A..........C..........   272
AF486858             241   ............................................................   300
AY574046             241   ............................................................   300

Reference(FJ237003)14417   ACGGAGCATCCATATTCTTTATTTGCCTATTCATCCACGTAGGCCGAGGTCTATACTACG   14476
KF185091             302   ...............................T.................C..........   361
KF185090             302   ............................................................   361
FJ190153             273   ...............................T.................C..........   332
Fj190159             274   ...............................T.................C..........   333
FJ190161             273   ...............................T.................C..........   332
AF486858             301   ............................................................   360
AY574046             301   ............................................................   360

Reference(FJ237003)14477   GATCCTATATATTCCTAGAAACATGAAACATTGGAGTAGTCCTACTATTTACCGTTATAG   14536
KF185091             362   ...............................C......A.T...................   421
KF185090             362   ............................................................   421
Fj190153             333   ...............................C......A.T...................   392
FJ190159             334   ...............................C......T.T...................   393
FJ190161             333   ...............................C......A.T...................   392
Af486858             361   ............................................................   420
Ay574046             361   ............................................................   420
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Plate-1. DNA sequence alignment of fragment of the Cytochrome b gene of selected wild boar, village pig and exotic pigs (Duroc, Land race & Hampshire) from Table 1. Dot (.) indicates nucleotide identity according to the reference sequence (FJ237003). Bold and highlighted nucleotide positions in the aligned sequence indicate the polymorphic sites of Indian wild boar from other pigs. The region used for restriction analysis (*StuI* - AGGCCT) is shown highlighted with red colour. Numbering is according to the gene bank sequence data. (FJ237003-European wild bear; KF185091-Indian wild pig; KF185090-Domestic pig; FJ190153, FJ190159, FJ190161- Sequences from pigs of Western Ghat forests; AF486858-Duroc; AY574046-Hampshire breeds)

*Dra*I enzyme and used European wild pig sequence as reference sequence to compare the sequences of village and exotic pigs [22]. Another Cytochrome b gene fragment (440 bp) of wild pig and domestic pig was sequenced previously to study the phylogenetics of the two sub species [1]. Though the DNA sequencing revealed unambiguous result, PCR-RFLP of 474bp Cytochrome b gene fragment could prove a quick and cost effective tool in forensic analysis of seized samples. The recent introgression of exotic genome due to the state sponsored programs conducted to upgrade the local population with imported semen or live animals of exotic breeds has diluted the gene pool of local breeds and precise breed specification of the local domestic remains questionable. 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. Owing to maternal inheritance, polymorphisms of mitochondrial genes provide means in species identification. The polymorphism observed in cytochrome b fragment sequence can be used to differentiate Indian wild boar from local domestic pigs, European wild pig and exotic pig breeds of Duroc and Hamshire.

Using the PCR-RFLP assay, we could resolve a forensic case registered under Damoh range, Madhya Pradesh Forest Department, (Sample No. 25F/8.01. 2013) in which confiscated meat samples of heart and muscle tissue matched with RFLP pattern of wild pig while PCR-RFLP pattern of two other pieces of seized meats under the same case matched with the domestic pig profile (Figure- 2).

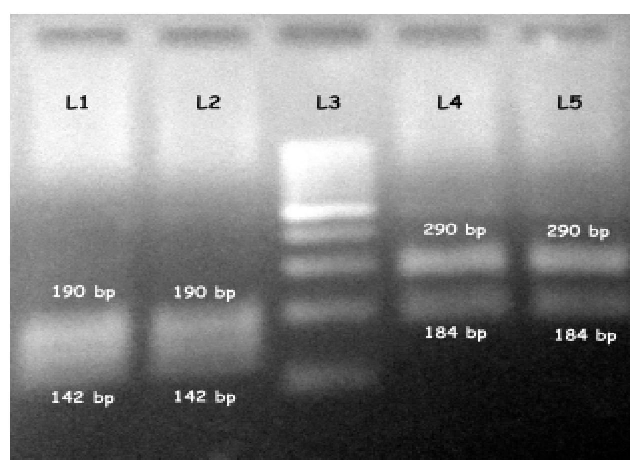

Figure-2. PCR-RFLP pattern produced by restriction digestion with *StuI* (L1, L2: Heart, Muscle sample, L3: 100bp Ladder, L4, L5: Meat sample)

Conclusion

In comparison to other phylogenetic marker, the cytochrome b gene demonstrates greater congruence

with conventional mammalian phylogeny and shows greater level of nucleotide variation in shorter sequence [23]. The restriction pattern obtained by *Stu*I enzyme on Cytochrome b gene fragment of 474 bp was found to be efficient for differentiating the samples of wild and domestic pig. The PCR-RFLP is a simple and very effective tool for differentiating the samples of both the sub species of pigs, which can help law enforcing agencies like Forest department, Police authorities to identify the sub-species which in turn would help in prosecution of the poachers.

Authors' contributions

KKJ designed the study. NR and KKJ analyzed and interpreted the results. ABS drafted and revised the manuscript for critical scientific corrections. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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