doi:10.5455/vetworld.2013.254-259

ORIGINAL RESEARCH

Characterization of 12S rRNA gene for meat identification of common wild and domestic small herbivores as an aid to wildlife forensic

Nidhi Rajput[1], A. B. Shrivastav[1], S.N.S. Parmar[2], Rakesh Ranjan[3], Sanjeev Singh[3] and E. Joseph[4]

1. Centre for Wildlife Forensic and Health, Madhya Pradesh Pashu Chikitsa Vigyan Vishwavidyalaya, Jabalpur, India
2. Department of Animal Breeding and Genetics, Madhya Pradesh Pashu Chikitsa Vigyan Vishwavidyalaya, Jabalpur, India
3. Animal Biotechnology Centre, Madhya Pradesh Pashu Chikitsa Vigyan Vishwavidyalaya, Jabalpur, India
4. Department of Veterinary Microbiology, Madhya Pradesh Pashu Chikitsa Vigyan Vishwavidyalaya, Jabalpur, India
Corresponding author: Nidhi Rajput, email: nidhi3rajput@gmail.com
Received: 24-08-20128, Accepted: 20-09-2012, Published online: 12-02-2013

How to cite this article: Rajput N, Shrivastav AB, Parmar SNS, Ranjan R, Singh S and Joseph E (2013) Characterization of 12S rRNA gene for meat identification of common wild and domestic small herbivores as an aid to wildlife forensic, *Vet. World* 6(5):254-259, doi:10.5455/vetworld.2013.254-259

Abstract

Aim: Chital and sambar are the common wild small herbivores, which are vulnerable to poaching for their meat. Many times poachers claim the wild meat to be that of goat or sheep. Hence, authentic evidences are required to stop such wildlife crime. The present investigation was carried out to study the species specific PCR-RFLP patterns for meat identification of chital and sambar and then differentiation from the meat of goat and sheep.

Materials and Methods: Extracted DNA from meat samples were subjected to PCR using the universal primers of 12S rRNA gene. The PCR products were subjected to RFLP and sequencing.

Results: The size of amplified PCR products was similar (440 bp) in each species and sequence alignment showed more than 89 % similarities among these species. However, phylogenetic analysis revealed that Chital and Sambar are in one cluster while Goat and sheep are in other cluster. To differentiate between species, restriction digestion of the PCR products was carried out to produce characteristic PCR-RFLP patterns for each species. Restriction digestion with *Rsa*I and *Alu*I enzymes produced distinct PCR-RFLP patterns that differentiated the meat of wild species (chital-sambar) from that of domestic species (goat-sheep). *Bsr*I restriction digestion revealed unique PCR-RFLP pattern in chital differentiating it from the meat of other three species. Restriction digestion with *Dde*I enzyme led to the production of distinct PCR-RFLP patterns for chital and sambar to identify their meat individually.

Conclusion: This study showed the effectiveness of 12S rRNA gene polymorphism in meat identification. The data can be used as evidence against the poachers to convict the wildlife crime in the court of law.

Keywords: 12S rRNA gene, meat identification, restriction fragment length polymorphism (RFLP), wild small herbivores, wildlife forensic

Introduction

India is one of the mega biodiversity country in the world. Wild species are important constituents of human ecosystem. Wild species are essential to maintain the health of forest and each has its own disposition in preserving the biodiversity [1]. Deer family plays an important role in the forest's natural cycle and is also gifted with elegant appearance. Deer family is a group of small herbivores including barahsingha, hangul, chital, sambar, barking deer, musk deer, brow antlered deer etc. In Central India, chital and sambar are the common wild small herbivores, which are found predominantly among the other species of deer family.

In the recent years, increased poaching of chital and sambar for their meat has raised an issue to the survival of these wild creatures. These species are listed in the Schedule III of Wildlife (Protection) Act, 1972 and their poaching is punishable. Inspite of prohibition, poaching of these wild species is frequent, which highlights the urgent need to monitor and take necessary steps to save these wild creatures [2,3]. Many times, when poachers are caught with the suspected meat, they claim the meat to be that of goat or sheep and lack of evidences has been identified as one of the major constraints to implement legal procedures in such cases.

The species identification of meat could be achieved using many methods such as Immunological tests, enzyme linked immunosorbent assays, sensory analysis, anatomical differences, histological differentiation of the hair that may possibly exist in the meat, properties of tissue fat, level of glycogen in muscle tissue, as well as electrophoresis and DNA hybridization [4-6]. However, these methods are less sensitive and often show cross reactivity in closely related species *i.e.* chital and sambar. Hence, characterization of species-specific molecular markers and designing of species-specific assays for identification of wild species are essential to produce evidences to convict the poachers of wildlife crime in the court of law. The modern advent of molecular DNA forensics now adds a new dimension to wildlife law enforcement, which has opened up the possibility of examining different biological samples *i.e.* meat piece, hairs, bone fragments, blood etc. [7].

Identification of the Indian wild species has been

conducted using the blood samples to establish a standard for species identification. Although, it was lacking to differentiate the meat of wild species from the meat of domestic species to generate the data, which could be applicable as convincing evidences against poachers to convict the wildlife crime in the court of law. Therefore, it is required to standardize a technique to identify the meat of wild species and differentiation from the meat of domestic species.

PCR based techniques have become a standard process in species identification using various biological samples. PCR uses the DNA, either mitochondrial or nuclear, to produce species-specific results. Though, mitochondrial DNA is preferred because there are approximately 2 to 10 copies of *mt*DNA in each mitochondrion and there are hundreds to many thousands of mitochondria per cell. Hence, it is possible to obtain a full *mt*DNA profile from more degraded or deteriorated samples or very small samples [8].

However, amongst the various PCR techniques, polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) technique has frequently been used and found effective in identification of meat species [9-13]. RFLP analysis is the first technique for DNA profiling which refers to the separation of two or more samples of homologous DNA molecules arising from differing locations of restriction sites and it is also useful to differentiate the closely related species. In this study, we utilized the polymorphism of 12S rRNA gene to identify the meat of Chital (*Axis axis*), Sambar (*Cervus unicolor*), Goat (*Capra hircus*) and Sheep (*Ovis aries*) using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique.

Materials and Methods

Samples collection and isolation of genomic DNA from the meat samples: Meat samples were collected from chital, sambar, goat and sheep irrespective of sex. The meat samples of chital and sambar were collected from the Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Jabalpur during post mortem examination, whereas of goat and sheep from the Military Butchery and local slaughter houses, Jabalpur. The samples were collected in a sterilized container and transported to the molecular diagnosis lab, Centre for Wildlife Forensic and Health, Madhya Pradesh Pashu Chikitsa Vigyan Vishwavidyalaya (M.P.P.C.V.V.), Jabalpur. The samples were stored at -20C for further analysis. DNA was isolated from the approximately 25 mg meat sample using DNeasy blood and tissue extraction kit (QIAGEN, GmBH, Hilden, Germany) according to the manufacturer's instructions. Quality check and quantification was checked by Nanodrop™ spectrophotometer and on 0.8% agarose gel electrophoresis. The samples with bright intense compact band on agarose gel with acceptable purity (i.e. ratio 1.7-1.9) were further used for PCR. The DNA concentration was determined and samples were diluted 10-30 times (approx. 30 ng/µl) with MiliQ water.

Amplification of the 12s rRNA gene: 12S rRNA gene was amplified from genomic DNA samples by polymerase chain reaction. The Universal primers of 12S rRNA gene (forward primer, 5'- CAA ACT GGG ATT AGA TAC CCC ACT AT-3'; reverse primer, 5'-GAG GGT GAC GGG CGG TGT GT-3') were used for the PCR. The reaction was carried out in 25 µl consisted of 90-100 ng template genomic DNA, 10 Pico mol of each primer and 2.5µl 1X Taq buffer without $MgCl_2$, 1.5µl 25mM $MgCl_2$, 2.5 µl dNTPs, 0.2 µl Taq DNA and rest is adjusted with nuclease free water. The PCR condition included an initial denaturation 94°C for 5 min followed by 35 cycles consisting of denaturation at 94ºC for 30 sec, annealing at 58ºC for 30 sec and extension at 72ºC for 1min. and final extension at 72°C for 10 min using Gradient Thermal Cycler™, Eppendorf, India. The PCR products were run on 1.5 % agarose gels and visualized under UV light.

PCR-RFLP of 12s rRNA: PCR products were digested using the restriction enzymes *Rsa*I, *Alu*I, *Bsr*I and *Dde*1 to generate the PCR-RFLP profile for identification of different meat species. RFLP was carried out in final reaction volume of 30µl in an incubator (37ºC for 16 hours). The reaction mixture contained 1µl restriction enzyme (*Fastdigest*, Fermentas Life Sciences), 3 µl buffer, 10 µl PCR product and 16 µl MilliQ water for each enzyme. To confirm the targeted RFLP digestion, 10µl of each RFLP product from each tube was mixed with 2µl of 6X gel loading buffer and electrophoresed along with DNA molecular weight marker (Gene Ruler, MBI Fermentas) on 2.5% agarose gel containing ethidium bromide (at the rate of 0.5 µg/1ml of gel solution) at 80V for 90 min in 0.5X TBE buffer.

Sequencing of PCR products: PCR products of chital, sambar, goat and sheep were subjected for both forward and reverse sequencing (Chromous Biotech Pvt. Ltd., Bengaluru, India). The samples were assembled using SeqScape v2.5 software programme.

Results

A distinct band of amplified PCR product of approx. 440 bp was observed in all the four species studied using gel electrophoresis. *Rsa*I enzyme digestion of 440 bp amplicon revealed two fragments of 400 bp and 40bp in goat and sheep whereas three fragments of 200, 150 and 90 bp in case of Chital and sambar (Fig 1a). The *Alu*I digestion also produced the two fragments of 340bp and 100 bp in chital and sambar, whereas two fragments of 240bp and 200bp in goat and sheep (Fig 1b). Digestion by *Bsr*I revealed two fragments of 250 and 190 bp in chital, while sambar, goat and sheep did not have any enzyme recognition site that is clearly shown on agarose gel (Fig 1c). The *Dde*I restriction digestion produced two fragments in all the animals but of different sizes. Fragments of 350 and 90 bp in goat-sheep, 300 and 140

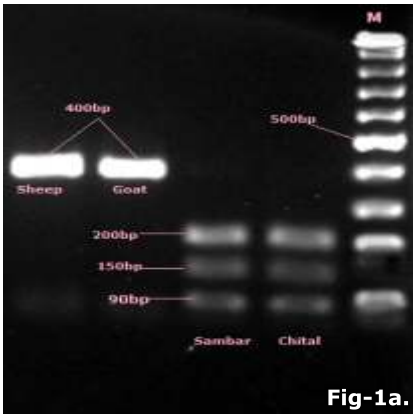

Fig-1a.

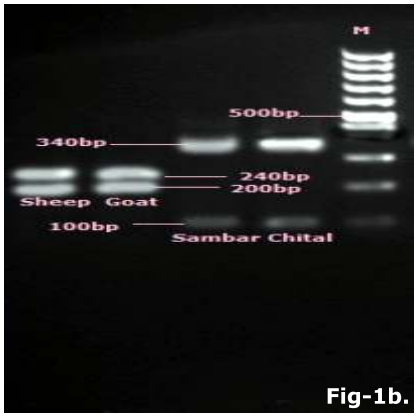

Fig-1b.

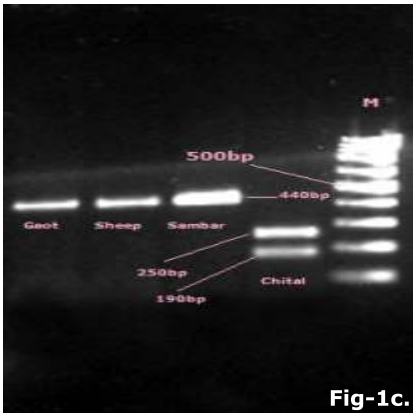

Fig-1c.

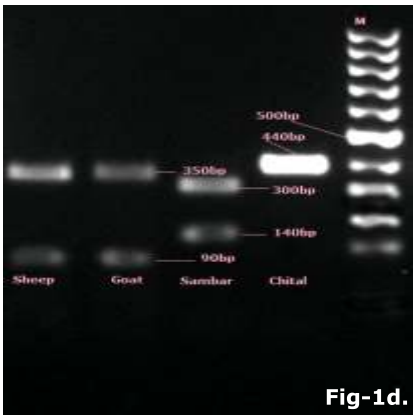

Fig-1d.

Fig-1a. Comparative PCR-RFLP patterns produced by *Rsa*I restriction digestion

Fig-1b. Comparative PCR-RFLP patterns produced by *Alu*I restriction digestion

Fig-1c. Comparative PCR-RFLP patterns produced by *Bsr*I restriction digestion

Fig-1d. Comparative PCR-RFLP patterns produced by *Dde*I restriction digestion

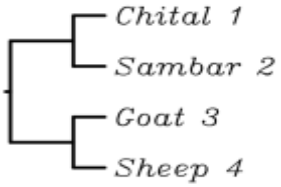

Figure-2. Rooted phylogenetic tree of Chital, Sambar, Sheep and Goat on the basis of 12S rRNA

Table-1. Percentage identity and diversity of Chital, Sambar, Sheep and Goat on the basis of 12S rRNA gene

% Diversity`	% Homology			
	Chital	Sambar	Goat	Sheep
Chital	97.00	93.00	94.00	
Sambar	03	---	93.00	93.00
Goat	07	07	---	100.00
Sheep	06	07	00	---

bp in sambar whereas 400 and 40 bp in chital were produced on agarose gel as (Fig 1d).

DNA sequence data from PCR products were found to correspond to the approx. size 440 bp. Sequence analysis of the nucleic acid sequences aligned using forward and reverse primers revealed variable percentage of homology (93 % to 100%), as depicted in Table-1. The Chital sequence showed 97, 93 and 94 % homology with sambar, goat and sheep respectively. Similarly, Sambar showed 93 % homology with goat and sheep. There was 100% homology is between goat and sheep 12srRNA sequences. The nucleotide sequence similarity and variation between these animals is shown in Plate-1. The phylogenetic analysis revealed that Chital and Sambar are in one cluster while Goat and sheep are in other cluster (Fig 2).

Discussion

Development of simple and authentic method for detecting the species origin of a wide variety of wild meat continues to be a major challenge before the wildlife forensic experts. In India, the prime concern remains with the identification of meat of wild herbivores. Common wild herbivores are chital and sambar, which are frequently being poached for their meat. Hence, proper meat identification methods are required especially for chital and sambar to prevent the poaching. Previously common method to identify the species of meat was based on immunological tests [15,16]. Immunological methods were found less reliable to differentiate between closely related species. PCR-RFLP is one of the PCR based techniques, which differentiates the two or more DNA samples by breaking them into fragments and separating on the basis of their lengths. This technique has been found effective and specific to identify the meat of different species.

Small amount of DNA (100 ng) was sufficient to produce PCR product and restriction digestion of this PCR product also led to production of distinct PCR-RFLP patterns. It proves that this method of PCR amplification will be useful in cases, where small amount of biological sample is available for forensic investigation. In the present study, PCR amplification of 12S rRNA gene revealed amplified product of 440 bp size in chital, sambar, goat and sheep species. It indicates the presence of mitochondrial DNA in all the samples. Gupta *et al.* [11] in chital and sambar and Chen *et al*. [12] in goat reported amplicon size of 440 bp, which is same as reported in the present study for

Plate-1. Alignment of approximately 440 bp sequences of 12S rRNA gene of Chital, Sambar, Sheep and Goat

"*"- Sign indicates nucleotides in that column are identical in all the sequences in the alignment, Yellow color indicates nucleotide variations in the sequences, Red color indicates forward and reverse primer location

chital, sambar, goat and sheep. Though, the PCR product of 456 bp size was also reported by Girish *et al.* [17] in mutton and chevon and Mahajan *et al* [13] in goat using 12S rRNA gene universal primers. The difference of approximately 16 bp in the PCR fragments of goat and sheep obtained in the present study suggests the possibility of insertion/ duplication of the sequence.

Sequence analysis of the mitochondrial 12S rRNA gene revealed 93 % to 100% homology in between species. This indicates close relationship between the species. However, phylogenetic analysis revealed difference between wild and domestic small herbivores.

To find out the differences of meat amongst four species *i.e.* chital, sambar, goat and sheep, four restriction enzymes, *Rsa*I, *Alu*I, *Bsr*I and *Dde*I, were used to observe the PCR-RFLP patterns. The restriction enzyme digestion produced specific PCR-RFLP patterns and on the basis of these patterns, meat samples of chital and sambar were identified and differentiated from the meat of goat and sheep.

PCR-RFLP patterns produced by *Rsa*I digestion clearly differentiated the meats of wild small herbivores (200+150+90 bp) from that of domestic small herbivores (400 bp). Though, Gupta *et al.* [11] reported fragments of 212 bp and 152 bp in chital and sambar after the *Rsa*I digestion of PCR products. No report is available on 12S rRNA gene polymorphism produced by this enzyme in goat and sheep. Similarly, PCR-RFLP patterns produced by *Alu*I digestion differentiated the wild meat (340 bp+100 bp) from the domestic meat (240+200 bp). Chen *et al.* [12] reported the PCR-RFLP patterns produced by this enzyme in goat having two fragments of 240 bp and 200 bp sizes, which is same as reported in the present study for goat. No report is available on 12S rRNA gene polymorphism produced by this enzyme in chital, sambar and sheep.

PCR-RFLP pattern produced by *Bsr*I restriction digestion was unique for chital (250+190 bp), which proves its valid application in the identification of chital meat. Therefore, *Bsr*I restriction enzyme is considered a suitable molecular marker to identify the chital meat positively. Gupta *et al.* [11] reported fragments of 252 bp and 179 bp in chital, while 431 bp in sambar after the digestion of PCR products using this enzyme. Though, no report is available on 12S rRNA gene polymorphism produced by this enzyme in goat and sheep.

Sambar is next to chital and also has been found as victim in many cases of wildlife crime. Thus, it becomes important to identify the meat of sambar and then its differentiation from the meat of chital. The PCR-RFLP patterns obtained from the restriction digestion with *Dde*I enzyme were distinctive for chital (400 bp) and sambar (300+140 bp) and found valid to identify the meat of these wild small herbivores

individually. On the basis of these patterns, the meat of two most common deer species can be identified and differentiated from that of their domestic counterparts (350+90 bp) to produce convincing evidence. Gupta *et al*. [11] reported single fragment of 379 bp in chital and 316 bp in sambar after the *Dde*I restriction digestion of PCR products. No report is available on 12S rRNA gene polymorphism produced by this enzyme in goat and sheep.

It is observed in the present study that the PCR-RFLP patterns produced by the four restriction enzymes *i.e. Rsa*I, *Alu*I, *Bsr*I and *Dde*I, can identify and differentiate the meat of chital and sambar from the meat of goat and sheep. Though, PCR - RFLP patterns obtained for chital and sambar were different from that reported by Gupta et al. [11], which suggests the possibility of insertion/ duplication of the sequence. Nevertheless, these PCR-RFLP patterns can be applied as evidence to convict the poachers of wildlife crime in the court of law.

Conclusion

DNA extracted from all the meat samples collected from chital, sambar, goat and sheep were able to amplify with the 12S rRNA gene universal primers, which revealed the PCR product size of 440 bp in all the species. Sequence analysis of 12S rRNA gene revealed 93% to 100% between these species. *Rsa*I and *Alu*I restriction digestion of PCR products revealed distinct PCR-RFLP patterns to differentiate between the meat samples of wild and domestic small herbivores *Bsr*I and *Dde*I restriction digestion of PCR products revealed species-specific PCR-RFLP patterns in chital and sambar. Hence, it is concluded from the results of present study that the meat of chital and sambar can be identified and differentiated from the meat of their domestic counterparts such as goat and sheep and these PCR-RFLP patterns can be used as evidence against the poachers to convict the wildlife crime in the court of law.

Author's contribution

This study was a part of Nidhi Rajput's M.V.Sc. & A.H. thesis and A. B. Shrivastav was her major advisor. All the authors contributed equally in designing of the research, analysis of the data, drafting and revision of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors are thankful to G.P. Mishra, Hon'ble Vice Chancellor and R.P.S. Baghel, Dean Faculty, M.P.P.C.V.V., Jabalpur for providing necessary facilities to carry out the research work. Author is also thankful to Dr. Sudhir Jain, Mr. Neeraj Tripathi and Dr. Kush Shrivastava for selfless help.

Competing interests

Authors declare that they have no competing interests.

References

1. Shrivastav, A.B. and Sharma, R.K. (2000) A manual of wildlife health management in protected areas. College of Veterinary Science & A.H., J.N.K.V.V., Jabalpur, M.P., India.
2. Mukherjee, S.K. (1995) Some thoughts on wildlife trade. *Cheetal,* 33:30-33.
3. Guha, S. and Kashyap, V.K. (2005) Development of novel heminested PCR assays based on mitochondrial 16S rRNA gene for identification of seven pecora species. *BMC Genet.*, 6:42-49.
4. Zade, N.N., Mandokhot, U.V., Goel, M.C. and Saxena, A.K. (2001) Species identification of meat by monoclonal antibody indirect enzyme linked immuno-sorbent assay. *Indian J. Anim. Sci.*, 71:38-39.
5. Abdulmawjood, A., Schnenbrucher, H. and Bulte, M. (2003) Development of a polymerase chain reaction system for the detection of dog and cat meat in meat mixtures and animal feed. *J Food Sci.*, 68:1757-1761.
6. Jha, V.K., Kumar, A. and Mandokhot, U.V. (2003) Indirect enzyme linked immunosorbent assay in detection and differentiation of cooked and raw pork from meats of other species. *J. Food Sci. Technol.*, 40:254-256.
7. Lorenzini, R., Posillico, M. and Petrella, A. (2004) Noninvasive genotyping of the endangered Apenine brown bear: a case study not to let one's hair down. *Anim. Conserv.*, 7:199 -209.
8. Tobe, S.S. and Linacre, A.M.T. (2008) A multiplex assay to identify 18 European mammal species from mixtures using the mitochondrial cytochrome b gene. *Electrophoresis,* 29(2):340-347.
9. Meyer, R., Chardonnens, F., Hubner, P. and Luthy, J. (1996) Polymerase chain reaction (PCR) in the quality and safety assurance of food: detection of soya in processed meat products. *Z. Lebensm Unters. Forsch.*, 203:339-344.
10. Alves, E., Castellanos, C., Ovilo, C., Silio, L. and Rodriguez, C. (2002) Differentiation of the raw material of the Iberian pig meat industry based on the use of amplified fragment length polymorphism. *Meat Sci.*, 61: 157-162.
11. Gupta, A., Patra, R.C., Das, D.K., Gupta, P.K., Swarup, D. and Saini, M. (2008) Sequence characterization and PCR-RFLP of the mitochondrial DNA 12S rRNA gene provides a method for species identification of Indian deer. *Mitochondr. DNA.*, 19(4):394 -400.
12. Chen, S.Y., Liu, Y.P. and Yao, Y.G. (2010) Species authentication of commercial beef jerky based on PCR-RFLP analysis of the mitochondrial 12S rRNA gene. *J. Genet. Genomics.*, 37: 763-769.
13. Mahajan, M.V., Gadekar, Y.P., Dighe, V.D., Kokane, R.D. and Bannalikar, A.S. (2011) Molecular detection of meat animal species targeting MT 12S rRNA gene. *Meat Sci.*, 88(1):23-27.
14. Sambrook, J., Frisch, E.F. and Maniatis, T. (1989) Molecular cloning: a molecular approach laboratory manual. New York: Harbour Laboratory Press.
15. Macedo-Silva, A., Barbosa, S.F.C., Alkmin, M.G.A., Vaz, A.J., Shimokomaki, M. and Tenuta-Filho, A. (2000) Hamburger meat identification by dot-ELISA. *Meat Sci.*, 56(2):189-192.
16. Necidova, L., Rencova, E. and Svoboda, I. (2002) Counter immunoelectrophoresis: a simple method for

the detection of species-specific muscle proteins in heat processed products. *Vet. Med. Czech.,* 47(5):143-147.
17. Girish, P.S., Anjaneyulu, A.S.R., Viswas, K.N., Anand, M., Rajkumar, N., Shivkumar, B.M., Patel, M. and Sharma, B. (2005) Meat species identification by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of mitochondrial 12S rRNA gene. *Meat Sci.,* 70(1):107-112.

****