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RESEARCH ARTICLE

MOLECULAR BIOLOGY

IDENTIFICATION OF FORENSIC CASE USING MOLECULAR MARKERS: A CASE STUDY OF HYAENA (*HYAENA HYAENA*)

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ABSTRACT

We describe a recent case in which an animal was killed, burnt and body parts were sent to the Institute for identifying species and the method used for extracting DNA from burnt tissues. Two mitochondrial gene cytochrome b and 16s rRNA were used for species identification. Sequence analysis revealed that the burnt sample was of a Hyaena (*Hyaena hyaena*), a Schedule III animal according to the Wildlife (Protection) Act 1972 of the Government of India.

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KEY WORDS

PCR amplification, chytochrome b, 16s rRNA, wildlife

INTRODUCTION

Conservation success of a species largely depends on control of illegal poaching and increased conviction rate of accused involved in poaching. Therefore, conviction under various Acts requires a need of identifying species from the biological materials seized from the accused. There are several challenges especially in DNA extraction and amplification while dealing wildlife offences in developing country as most of the biological material are either putrefied or burnt beyond recognition.

Nowadays molecular markers are considered as an advanced technique in evolutionary history. The DNA sequences of single gene or more, in part or whole, comprise the most common type of sequences used in molecular wildlife forensics. Analysis of mtDNA markers have widely been used for species identification in forensics laboratory by PCR amplification due to its high copy number of mt DNA per cell compare to nuclear DNA and lack of recombination. Mitochondrial DNA is haploid maternally inherited, similar in nucleotide sequences among co- specifics from the same geographic region and more suitable for species identification, in contrast to hypervariable DNA fingerprints.

The cytochrome b (Cyt b) and 16s rRNA genes are located in the mitochondrial genome and their highly conserved nucleotide sequences are known to be species-specific by Hsieh et al.[1]. Cyt b gene is widely analysed for species-specific identification, phylogenetic analysis and forensic investigations by Kocher et al.[2], Hsieh et al.[3] and Wan et al.[4]. Large ribosomal 16s rRNA gene has also been used widely for species identification, as well as for phylogenetic analysis by Carrera et al.[5] and Stubbs et al.[6]. Therefore, partial fragments of cytochrome b gene by Verma et al.[7] and 16s rRNA gene by Mitchell et al.[8] of mitochondrial DNA were chosen for identifying species from a burnt case property referred to the Institute.

MATERIAL AND METHODS

2.1. Case Property and Reference Sample

The Divisional Forest Officer, (Madhya Pradesh, India) had captured some completely burnt (Fig. 1, a and b) sample with a suspicion that a wildlife crime was committed. The sample was sent to our laboratory to identify the species. Suspected species were either leopard or hyaena. We examine the availability of cyt b and 16s rRNA gene sequences of case sample at NCBI (National Center for Biotechnology Information), But we did not find any 16s rRNA gene sequences for Hyaena. Therefore, we separately took a known meat sample of Hyaena (*Hyaena hyaena*) as reference for species identification of case species.

Fig 1(a)

Fig 1(b)

Figure 1 (a) and 1(b)
Condition of sample received in the lab

2.2. DNA Isolation

The material sent to us was so burnt that it was very difficult to get sample suitable for DNA isolation. DNA was extracted three times but we could not get it. So burnt skull present with the case property was broken, washed with bleach and distilled water. Surface of bone peaces was removed with a sand disk. The cleaned bone was then powdered with the help of liquid nitrogen in a sterile pastel motor. Powdered bone was suspended in 1.5 ml tube with 0.5mm EDTA (ph 8.0) for three to four days at room temperature with simultaneous exchange of EDTA in three to four hours. After the decalcification in three to four days, the supernatant was removed after centrifugation. Obtained pallet was then used for the DNA extraction. The commercially available DNeasy Tissue Kit (QIAGEN, Germany) was used to extract DNA from this sample. After sequencing, we could not get the data for 16s rRNA and 12s rRNA sequences in NCBI. So we have taken one reference sample from zoo (natural death) and that was also extracted as a known reference meat sample of Hyaena (*Hyaena hyaena*) using commercially available DNeasy Tissue Kit (QIAGEN, Germany).

2.3. PCR Amplification

Isolated DNA was used as a template to amplify cytochrome b and 16s rRNA genes using the universal primer:
mcb398,
5'-TACCATGAGGACAAATATCATTCTG-3'
and
mcb869,
5'-CCTCCTAGTTTGTTAGGGATTGATCG-3' by Verma et al. (6)
and
5'-CGCCTGTTTATCAAAAACAT-3'
and
5'-CTCCGGTTTGAACTCAGATC-3'
by Mitchell et al. (8) respectively.

Polymerase chain reaction (PCR) was performed in (M J Research PTC 200 Peltier Thermo Cycler) a final volume of 25 µl reaction volume containing 1x PCR Buffer (10mM Tris-HCl, pH 8.3 at 25^0C; 50mM KCl, SIGMA, USA); 5mM $MgCl_2$ (SIGMA, USA); 10 mM dNTPs (SIGMA, USA); 5pmol of each primer (Sigma-Genosys Ltd., USA); 1U Taq polymerase (MBI Fermentas) and varying quantities (50-100ng) of DNA to ensure that the optimum amount for amplification would be present. Amplification conditions were 94^0C for 2min followed by 30 cycles at 94^0C for 1min, annealing temperature (Ta) for 1min and 72^0C for 1min, with final extension of 72^0C for 10 min. Amplified PCR products were purified with a QIAquick spin purification kit (QIAGEN, Germany). Cycle sequencing PCR was performed for these purified PCR products with their respective primers following the suggested composition of master mixture from Applied Biosystems 3130 Genetic Analyzer protocol. Cycle sequencing PCR products were cleaned up by using QIAGEN Dye Spin kit**.** These products obtained were sequenced (Applied Biosytems 3130 Genetic Analyzer) on both strands in triplicate (from different amplified product) and were checked and edited. Sequence was searched with GenBank database (National Center for Biotechnological Information, USA: NCBI) using BLAST program.

3. RESULT AND DISCUSSION

DNA technology is being a powerful tool for species identification basically involves sequence analysis of mt DNA. The universal primers for mitochondrial cyt b gene (Kocher et al., [2.] and 16s rRNA can amplify corresponding regions from a wide variety of organisms. The cytochrome *b* gene is the most widely used gene for phylogenetic work and species identification because it evolves slowly in terms of non-synonymous substitutions, the rate of evolution in silent positions is relatively fast and conserved enough for clarifying deeper phylogenetic relationships. The partial sequences of 16s rRNA gene is used for species identification because it showed a high degree of similarity to corresponding sequences in GenBank database. Thus the partial 16s rRNA sequences can used for species identification and authentication of animal tissues. The richness of the cytochrome b and 16s rRNA gene database also makes it an excellent molecule of choice for identification and species authentication purposes.

DNA was extracted three times and fragment sizes were from 50bp to 600bp. By this method, we are getting good size base pair as we are doing lot's of case regarding bone as our laboratory is wildlife forensic lab. After sequencing, the most significant alignment for the cytochrome b sequences generated with the DNA obtained from burnt sample was with cytochrome b (bits score 790, *E*-value 0.00) gene sequence of hyaena (NCBI accession no. AY048788) but we could not get the sequence for 16s rRNA and 12s rRNA in NCBI. So we used one known reference sample of hyaena which we already had from the zoo (as our laboratory is collecting the sample from different zoo which is found natural death in zoos). After getting the known sequences of cytochrome b gene, 16s rRNA and 12s rRNA of reference sample, our case sample matched to hyena using our reference sample. We have submitted the sequences for cytochrome b (EF107524) and 16s rRNA in NCBI (Accession No. EF202837 and EF107510). We also compared the sequences of all three species of h*yaenidae* family for the cytochrome b region but for 16s rRNA and 12s rRNA we could not find the sequences. Pair wise comparisons indicate a differentiation among all investigated species from Hyaena by minimum of 24 (brown hyaena) nucleotide in cytochrome b (Table 1), 41 (leopard) nucleotide variation (Table 2) in 16s rRNA and 32 (tiger) nucleotide variation (Table 3) in 12s rRNA. The cytochrome b, 16s rRNA and 12s rRNA sequences obtained from case had maximum similarity 99% (Table 4), 100%

(Table 5) and 100% (Table 6) respectively with known Indian Hyaena (*Hyaena hyaena*). Neighbor- joining tree obtained using Kimura's two-parameter (Table 7) method generated to show relationship among three species of family hyaenidae and other species of the mitochondrial DNA cytochrome b gene. All the three species of hyaenidae is with 100/70/100 bootstrap values. But we could not find Neighbor- joining tree for 16s rRNA and 12s rRNA bec. We could not find the sequences for all three species of hyaenidae in NCBI data.

Table 1

Number of variable site (above diagonal) and percent similarity (below diagonal) amongst cytochrome b sequences with other sequences of reference animals (From NCBI GenBank).

	Case723	Hyaena	B. hyaena	S. hyaena	Buffelo	Wolf	Jackal	Leopard	D. cat	Tiger
1. Case723	_	1	25	36	62	70	67	66	65	64
2. Hyaena	99	_	24	36	63	69	67	65	64	64
3. Brown Hyaena	92	93	-	33	70	65	61	58	58	62
4. Spotted Hyaena	89	89	90	-	69	75	68	63	62	66
3. Buffelo	82	82	80	80	_	64	64	69	68	70
4. Wolf	80	80	81	78	81	_	26	69	70	62
5. Jackal	81	81	82	80	81	92	_	66	69	66
6. Leopard	81	81	83	82	80	80	81	_	37	32
7. Domestic cat	81	81	83	82	80	80	80	89	_	45
8. Tiger	81	81	82	81	80	82	81	90	87	_

Table 2

Number of variable site (above diagonal) and percent similarity (below diagonal) amongst 16s rRNA sequences with other sequences of reference animals (From NCBI GenBank)

	Case	Hyaena	Buffalo	Wolf	Jackal	Leopard	Domestic cat	Tiger
1. Case	_	0	52	40	38	41	33	32
2. Hyaena	100	_	52	40	38	41	33	32
3. Buffalo	83	83	_	40	39	66	51	49
4. Wolf	87	87	87	-	6	45	39	38
5. Jackal	87	87	87	98	-	46	38	39
6. Leopard	87	87	81	85	85	-	22	18
7. Domestic cat	89	89	83	87	87	92	-	14
8. Tiger	90	90	84	87	87	94	95	-

The 16s rRNA sequences obtained from case is 100% (zero variation) similar to Hyaena 16s rRNA sequences, indicating the source of material is Hyaena species.

Table 3

Number of variable site (above diagonal) and percent similarity (below diagonal) amongst 12s rRNA sequences with other sequences of reference animals (From NCBI GenBank).

	Case	Hyaena	Buffalo	Wolf	Jackal	Leopard	Domestic cat	Tiger
1. Case	_	0	52	40	38	41	33	32
2. Hyaena	100	_	52	40	38	41	33	32
3. Buffalo	83	83	_	40	39	66	51	49
4. Wolf	87	87	87	-	6	45	39	38
5. Jackal	87	87	87	98	-	46	38	39
6. Leopard	87	87	81	85	85	-	22	18
7. Domestic cat	89	89	83	87	87	92	-	14
8. Tiger	90	90	84	87	87	94	95	-

The 12s rRNA sequences obtained from case is 100% (zero variation) similar to Hyaena 12s rRNA sequences, indicating the source of material is Hyaena species.

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Table 4

Comparison of the cytochrome b DNA sequences of tissue of an unknown origin collected from burnt sample. Dot denotes nucleotide identical in all species

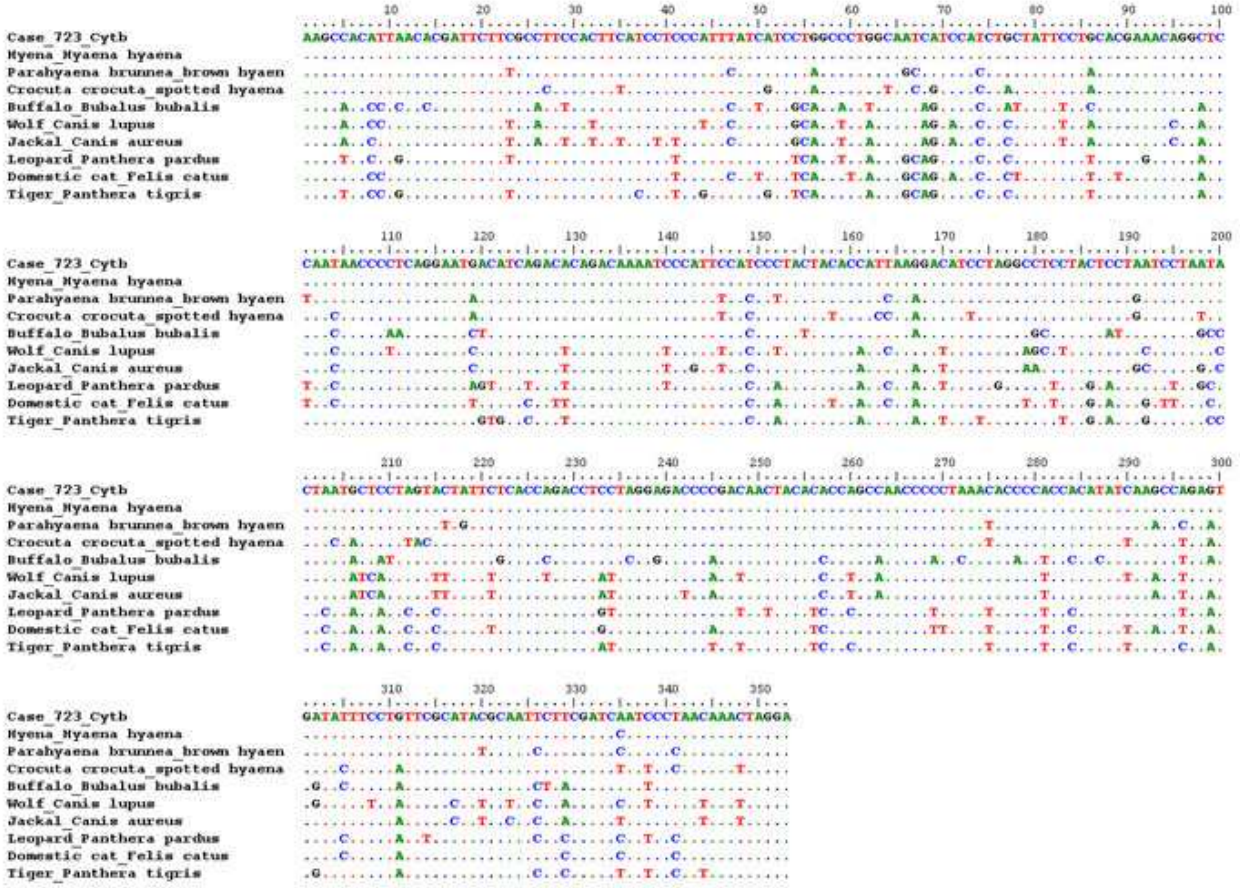

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Table 5

Comparison of 16s rRNA sequences of tissue of an unknown origin collected from burnt sample. Dot denotes nucleotide identical in all species.

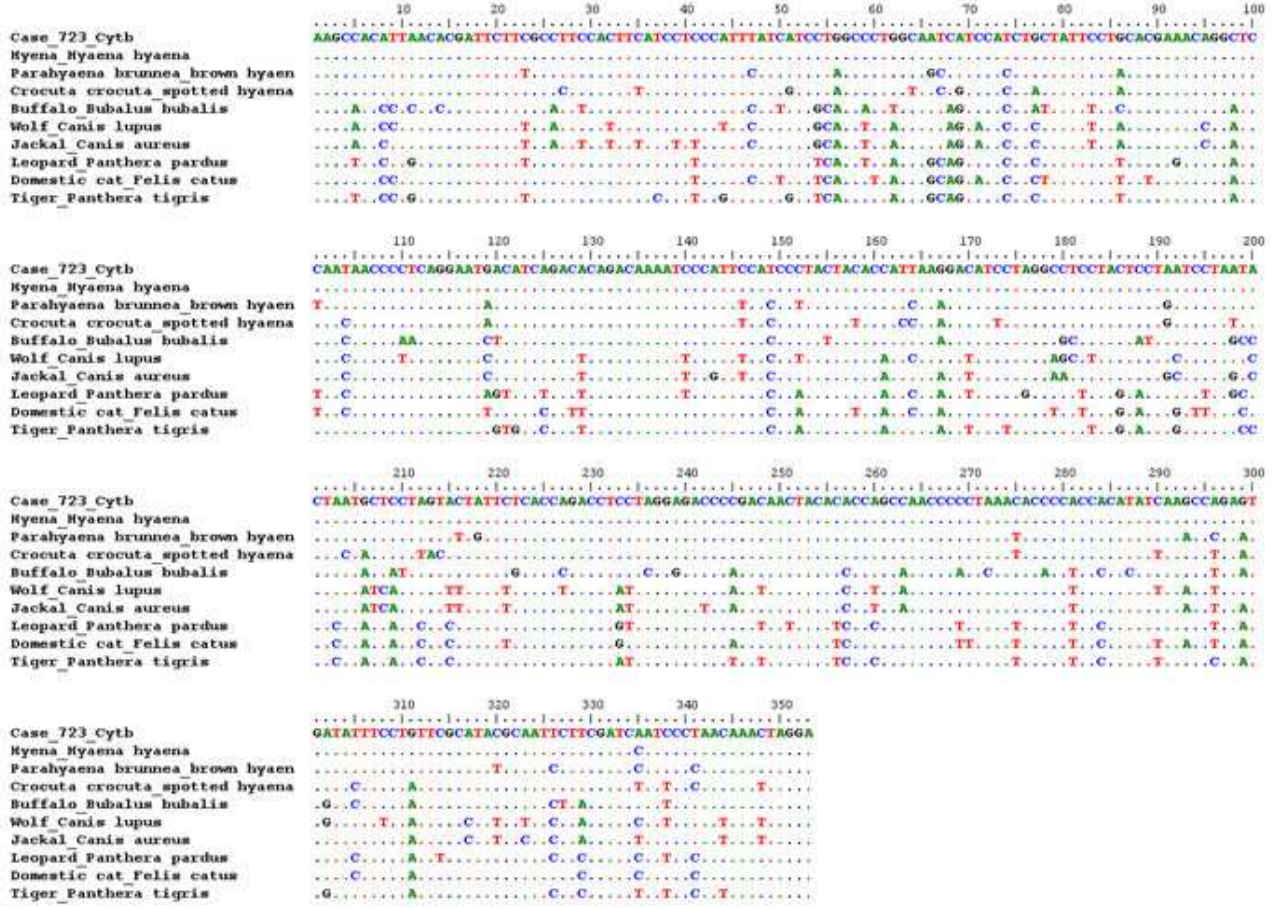

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Table 6

Comparison of 12s rRNA sequences of tissue of an unknown origin collected from burnt sample. Dot denotes nucleotide identical in all species.

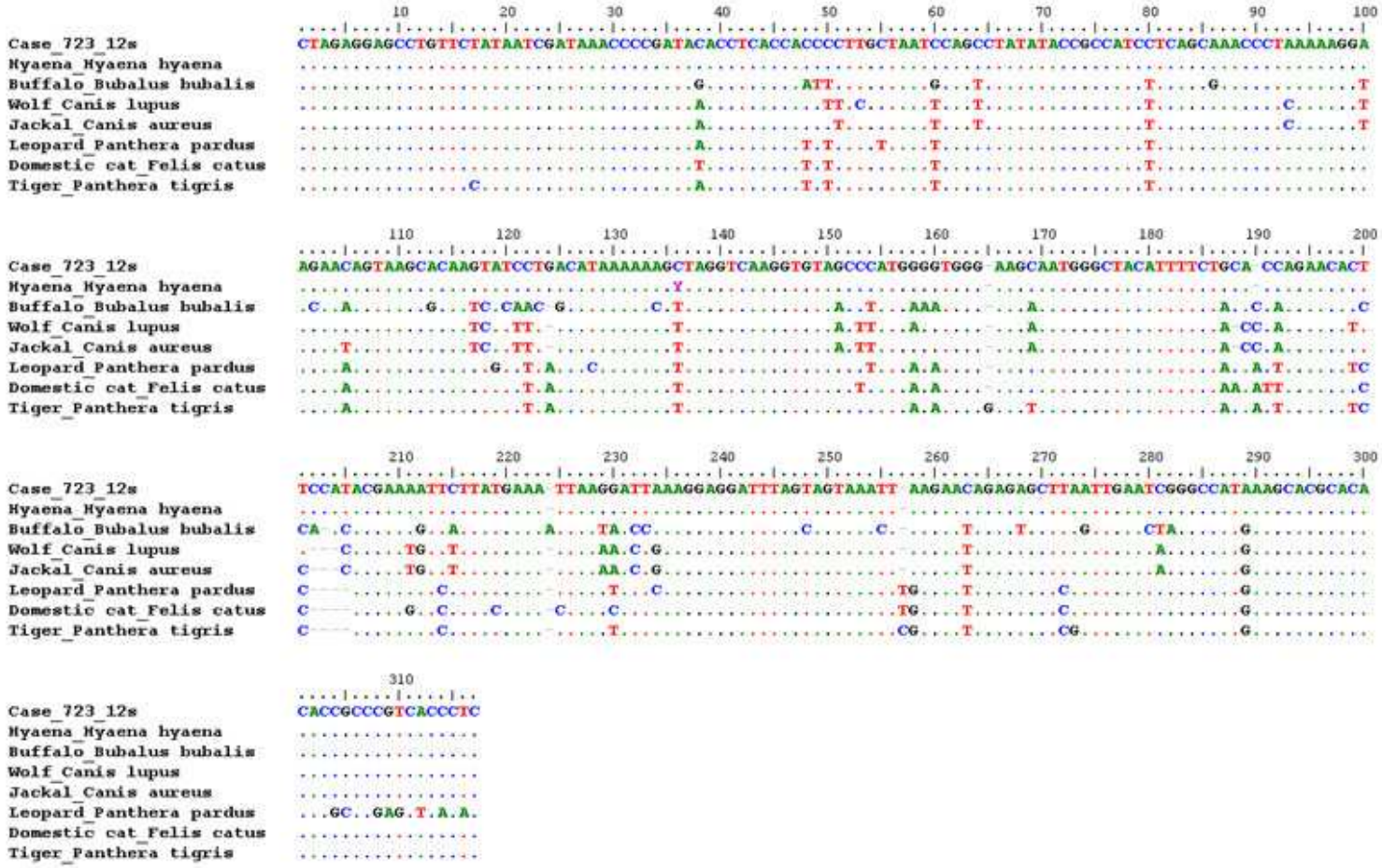
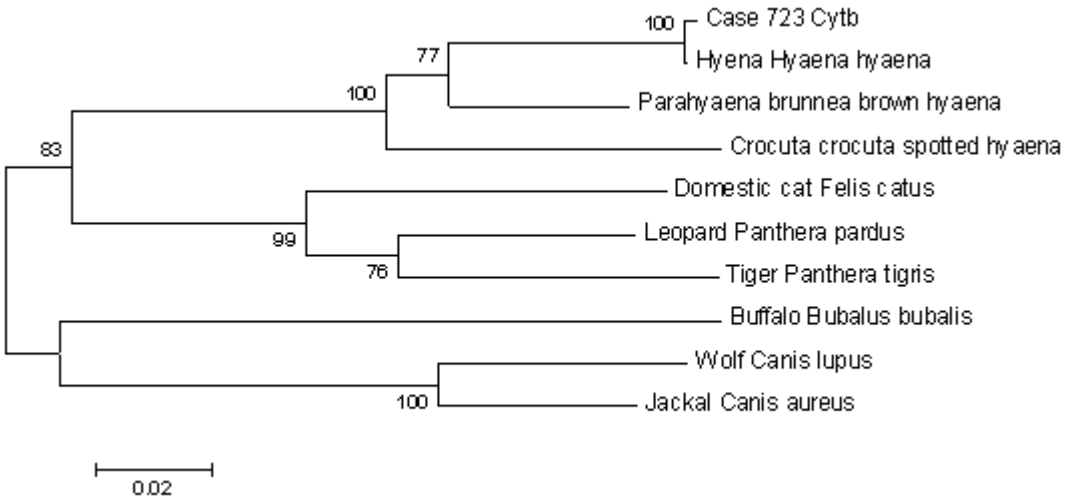


Table 7

Neighbor- joining tree showing relationship among three species of family hyaenidae and other species of the mitochondrial DNA cytochrome b gene.



Species identification based on cytochrome b, 16s rRNA and 12s rRNA gene are very accurate methods to identify species from confiscated samples. On the basis of sequence alignment and a score table for the case sample based on cytochrome b and 16s rRNA and 12s rRNA (*Hyaena hyaena*) in comparision with other species examined. In NCBI there is no data base for the Indian species. This study includes the data base for the Indian Hyaena to confiscate the cases. Therefore, we conclude that the sample sent to us under above case is of Hyaena (*Hyaena hyaena*) which is a Schedule III species animal according to the Wildlife (Protection) Act 1972 of the Government of India. Our finding also reveals that the sample taken from surface of skull as in this study among burnt wildlife sample may be suitable for species identification.

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