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A Pioneer Case Study on Identification of Infant Rhinoceros Horn

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Abstract

Objective: To investigate the authenticity of infant Rhino horn sample from morphometric, microscopic, genetic and phylogenetic studies.

Methods: A suspected rhino horn sample (11 cm × 13 cm) was sent to the laboratory for identification. Relevant morphometric data of sample were taken. Similarly, established methods for microscopic examination of rhino horn were adopted. Using standard universal primer, a part of Cytochrome b (Cyt b) and 12S rRNA was amplified from sample, sequenced and aligned using BLAST of the GenBank (NCBI) and in-house reference database available at Wildlife Institute of India, Dehradun, India for decisive confirmation of species.

Results: Morphometric analysis showed the sample was oval, dome shaped with a bony part which was supported by upper skin. Presence of filamentous units is the microscopic identifying feature of genuine rhino horn. However, microscopic examination showed very unclear filamentous units because of the immature nature of sample. Mitochondrial sequence data revealed the source of suspected sample was of a one-horn Rhinoceros (*Rhinoceros unicornis*). Similarly, NJ method for phylogenetic analysis for both Cytb and 12S rRNA identified sequence similarity with *Rhinoceros unicornis*.

Conclusion: Even though the morphometric and microscopic data do not reveal much about the infant rhino horn, mtDNA sequencing used in this pioneer study can be applied in the wildlife forensic for identification of such parts in future and data used as evidence against the poachers in the court of law.

Keywords: Infantile rhino horn; Forensic science; Filamentous unit; Cyt b; 12S rRNA; BLAST; Neighbour-joining method

Introduction

Wildlife and their products represent the third greatest illegal traffic after drugs and arms [1]. Illegal poaching are threats to a wide range of endangered species around the world [2]. Many species are protected both at a national and international level and their conservation requires control of illegal poaching [3]. Concept of "species" is perhaps the most debated subject in evolutionary biology [4]. But accurate identification of the species from confiscated biological material is of paramount importance to the investigation of crime in forensics [5], fixing the accurate wildlife offence [6] and so as to prove the conviction of crime by offenders [7]. The test employed depends on the material seized and the available techniques [3]. Morphology and microscopy are the natural starting points in identification [8]. However, the morphological examination has some limitations especially when material is present as powders, potions, oils [3], examination from old, degraded [9], partial, small parts [7] or processed, tanned, finished wildlife products [10]. Currently, DNA sequencing analysis is the proven and most reliable method used for species identification from confiscated biological samples [7]. A number of case reports have shown the usefulness of DNA analysis for solving cases including endangered species [11-20].

Material suspected of originating from Rhinoceros is frequently seized by forensic organizations especially in parts of Asia where the

rhinoceros horns are used traditionally [21]. A suspected rhino horn sample was sent to our laboratory for identification. A variety of methods are available to characterize a genuine rhino horn and its products [22]. However, usual morphological and microscopic techniques were inappropriate because of the nature of suspected sample. In this pioneer article, we report the successful identification of ambiguous infant rhinoceros horn through the combined application of mitochondrial Cytochrome b (Cyt b) and 12S rRNA gene sequence analysis and microscopic examination. The procedure can be used by scientific community and data used as evidence against the poachers to convict the wildlife crime in the court of law.

One-horned rhinoceros (*Rhinoceros unicornis*) inhabiting primarily in India and Nepal is included in Appendix I of the CITES. This species is also listed in schedule I of the Nepal Government's National Parks and Wildlife Conservation Act 1973 and in Indian Wildlife (Protection) Act 1972. As per provision in the Act, any person found guilty of possessing, selling or buying Rhinoceros horn or other body parts is subject to a fine of NRs. 50,000 to 100,000 (1USD~105NRs) or 3-15 years of imprisonment or both. Rhino horn seemed to be world's most valuable substance and thus its illicit trade has been steadily increasing with different purposes including ritual, medicinal and recreational activities [22]. In Nepal, horn bearing nasal part below the three years age of Rhinoceros is locally called Brahma-khaag (Brahma-Hindu creator god, Khaag-Rhino horn) which has notable religious value.

Methods

Case history

The Department of National Parks and Wildlife Conservation, Babarmahal, Nepal had received a suspicious rhino horn sample. The sample was sent to our laboratory to identify the confiscated samples and if possible species identification also.

Morphometric examination

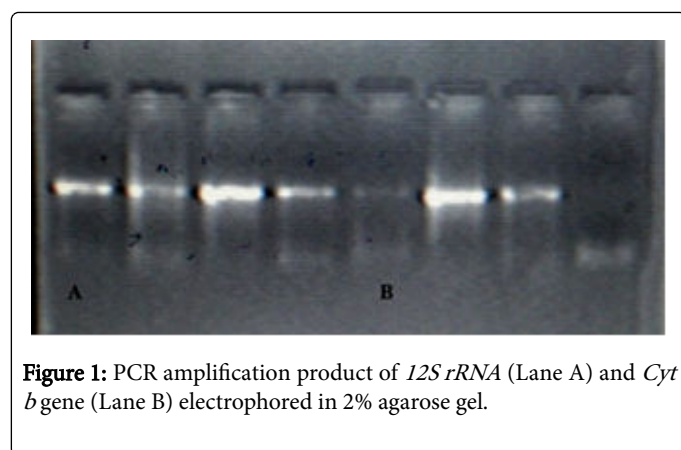
The overall shape, surface texture and configuration of sample were studied and measurements taken.

Microscopic examination

Thin transverse sections from the apex and margin of the sample skin were randomly taken with the help of a sharp razor blade. The sections were cleansed with xylene, dried, mounted in a clean microscopic slide with DPX and observed under 200X using light microscope (Olympus CX41 RF, Japan). A previously developed methodology was adopted to study the internal microscopic structure of hair [23].

DNA extraction and PCR

Approximately 100 mg skin piece was used for the extraction of DNA using standard protocol [24] in 100 μ L extraction volume. The isolated DNA was amplified using the universal PCR primers: mcb398, 5'-TACCATGAGGACAAATATCATTCTG-3' and mcb869, 5'CCTCCTAGTTTGTTAGGGATTGATCG-3' [5] as well as conserved primers: 12S rRNA-F_L1091, GCTTCAAACCTGGGATTAGATACCCAC and 12S rRNA-R_H1478, TGACTGCAGAGGGTGACGGGCGGTGTGT [25] targeting the mitochondrial *Cyt b* and *12S rRNA* gene respectively. The use of universal primers minimizes the effort of researcher, since it can be applied for PCR amplification to the DNA of all species without prior information of the victim species [26].



The PCR reaction mixture was prepared in a dedicated laminar hood. PCR amplification was performed in a Veriti thermal cycler (Applied Biosystems, Singapore) in a final volume of 10 μ L containing 1 μ L of the extracted DNA, 0.2 μ L of forward primer (5 pmol) and 0.2 μ L of reverse primer (5 pmol), 1 μ L of 10X buffer, 0.6 μ L of Magnesium chloride (3.0 mM), 1 μ L Bovine serum albumin (2 mg/ml), 0.2 μ L dNTPs (2.5 mM), 0.2 μ L Taq polymerase (5 units/ μ L) and 5.6

μ L Double distilled water. The PCR conditions were as follows: an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 35 seconds, primer annealing at 53°C for 45 seconds, primer extension at 72°C for 40 seconds. The final extension was at 72°C for 10 min. Negative PCR control was subjected to amplification. The PCR products obtained were electrophoresed on 2% agarose gel, stained with ethidium bromide, and visualized under a UV transilluminator to assess amplification (Figure 1).

Post PCR clean-up and DNA sequencing

The PCR product obtained was treated with Exonuclease I (Exo I) and Shrimp Alkaline Phosphatase (SAP) to clean the unused primers and dNTPs. 1.5 μ L of PCR product was directly incubated with 0.5 μ L ExoSAP at 37°C for 20 min. followed by inactivation of enzymes at 85°C for 15 min. The PCR product after Exo-I and SAP treatment was cycle sequenced. PCR amplification was performed in a Veriti thermal cycler in a final volume of 10 μ L containing 2.0 μ L of ExoSAP and PCR products, 1 μ L of Big dye 5X sequencing buffer, 0.5 μ L of Big dye RR mix v 3.1, 0.2 μ L of Primer (Forward only) and 6.3 μ L of double distilled water. The PCR conditions were as follows: an initial denaturation at 96°C for 5 sec, followed by 28 cycles of denaturation at 96°C for 10 seconds, primer annealing at 50°C for 5 sec, primer extension at 60°C for 4 min. Sequencing reaction products were purified by Sodium acetate ethanol precipitation method and detected by an ABI 3500xL Genetic Analyzer (Applied Biosystems, USA).

Data analysis

Quality of sequences was determined using Sequencing Analysis software v 6.0 (Applied Biosystem). Further Multiple Sequence Alignments (MSA) was performed by CLUSTAL W algorithm implemented in BIOEDIT version 7.0.9.0 [27]. The obtained consensus sequence was compared with sequences available on public domain at GenBank (<http://blast.ncbi.nlm.nih.gov/>) using BLAST [28] and in a "in-house database" available in Wildlife Institute of India, Dehradun, India. Phylogenetic trees including all the aligned sequences for the *Cyt b* and *12S rRNA* genes were generated based on Kimura 2 parameter model for nucleotide substitution with 1000 bootstrap values replications using the Neighbor-joining (NJ) method [29] as implemented in MEGA v 5.1 software [30].

Results and Discussions

Morphometry

Suspected sample was oval, dome shaped, bony part supported by upper skin (Figure 2a and 2b). The measured size, height and circumference were 11 \times 13, 4.0 and 25 cm respectively. Periphery of the horn was rough while the centre was smooth. Maximum thickness of skin measured was 0.81 cm. Ventral surface had frequent bony elevations (Figure 1c). Fewer black, short, coarse hairs were observed at the margins only (Figure 1b). Overall, these findings indicated that sample was skull or skull part of a big mammal. Hence, possibility of rhinoceros skull part, especially nasal part of immature ones was not debarred. Rhino horn is anchored to the dermis covering the frontal and nasal bones [31]. Authentic rhinoceros skull was unavailable in the laboratory. Thus, a definite conclusion about the sample was not possible from the morphological data only. Morphological examination has limitations especially when reference sample from known source is unavailable [6]. Identification of the suspected

rhinoceros material in the absence of morphological uniqueness is problematic [21]. No any visual evidence of a Rhino horn was observed in the sample. This is probably because a rhinoceros calf is born hornless [32]; pierce the skin at 5 weeks, 39 mm long at 3 months and 100 mm long at 7 months [33].

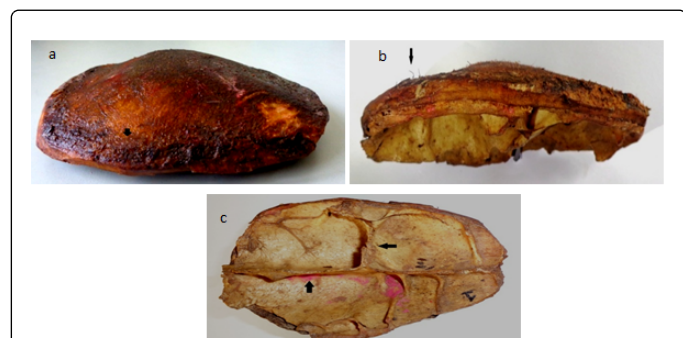


Figure 2: Dorsal (a), Lateral (b) and ventral (c) view of sample. Section site (a), hair (b) and bony elevations (c) indicated by arrow.

Microscopic findings

The occurrence of filamentous units and laminae is the microscopic identifying feature of genuine rhino horn [22]. In microscopic examination, section from margin of sample showed indistinct filamentous units, laminae with larger filamentous spaces (Figure 3).

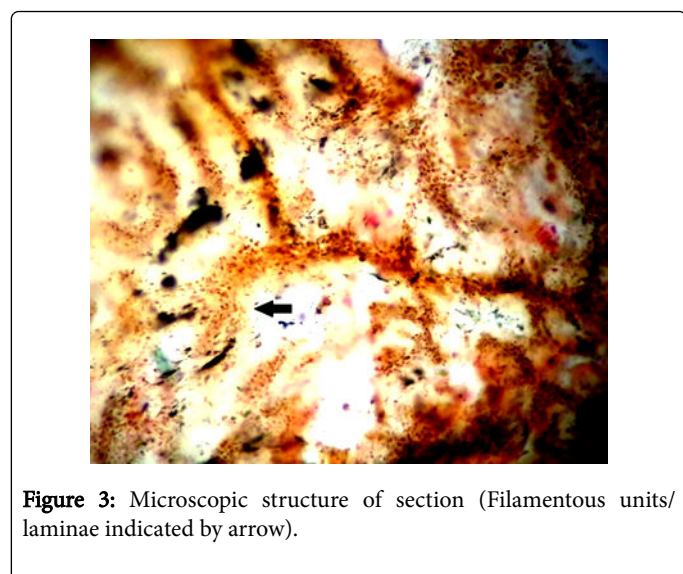


Figure 3: Microscopic structure of section (Filamentous units/laminae indicated by arrow).

This is probably due to immaturity of horn. Immature horns have greater filamentous units space in comparison to matured rhino horn [22]. Presence of these microscopic identifying features over the skin surface strongly indicated sample as an infant rhinoceros nasal part that bears horn.

Species identification based on hair characteristics has been widely used in dealing the wildlife offence [34]. However, medulla and other specific characteristics were not observed in the hair except profound pigment granules (Figure 4). The maximum measured length and diameter of hair was 5 mm and 150 µm respectively.

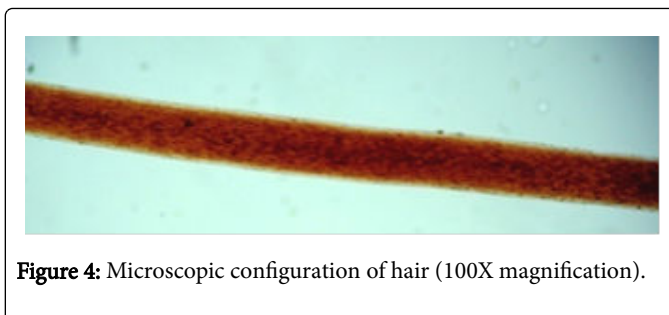


Figure 4: Microscopic configuration of hair (100X magnification).

Genetic analysis

Mitochondrial DNA (mtDNA) is most commonly used in species identification and attracts researchers because of its compact size, high copy number per cell, maternal inheritance, absence of introns and pseudogenes and fast evolutionary rate [35-38]. Cyt b, cytochrome oxidase I (COI), two ribosomal RNA (12S rRNA and 16S rRNA), the control region (D-loop) and subunits of mitochondrial encoded NADH dehydrogenase gene are the widely deployed mitochondrial gene for species identification in wildlife forensic [39].

Species/ Subspecies	Cyt b (187 bp)		12S rRNA (140 bp)	
	Accession Number	Similarity (%)	Accession Number	Similarity (%)
<i>Rhinoceros unicornis</i>	JN935370.1	100	AY739618.1	100
<i>Rhinoceros sondaicus</i>	AJ245725.1	87	AJ245724.1	98
<i>Rhinoceros sondaicus sondaicus</i>	-	-	AY739620.1	98
<i>Rhinoceros s. annamiticus</i>	-	-	AY739619.1	98
<i>Diceros bicornis minor</i>	-	-	FJ608808.1	98
<i>Coelodonta antiquitatis</i>	GU371439.1	86	-	-
<i>Dicerorhinus sumatrensis</i>	AJ245723.1	85	AJ245722.1	97
<i>Dicerorhinus s. sumatrensis</i>	-	-	AY739616.1	97
<i>Ceratotherium simum simum</i>	FJ619038.1	85	FJ608805.1	96
<i>Ceratotherium simum cottoni</i>	FJ619039.1	84	FJ608806.1	97

Table 1: Details of used species/subspecies and their GenBank Accession number of *Cyt b* and *12S rRNA* gene.

The central concept in species identification is to match the sequence of the evidence item to a reference sequence, either through DNA sequence similarity searches [40] or by phylogenetic reconstruction [41]. In sequence matching approach, a measure of the similarity between sequences is calculated and the most similar species is attributed to the sample [42,43] A 187 bp consensus sequence of Cyt

b was obtained from confiscated sample and a sequence similarity search performed in GenBank provided the highest similarity (100%) with *Rhinoceros unicornis* (NCBI Accession JN935370) i.e. an Asiatic one-horned Rhinoceros, whereas, it had 87% sequence similarity with *Rhinoceros sondaicus* (NCBI Accession: AJ245725) followed by other Rhinoceros species/subspecies with a decreasing sequence similarity (Table 1). Similarly, 140 bp consensus sequences of 12S rRNA was obtained which also showed highly similarity (100%) to the *Rhinoceros unicornis* (NCBI Accession: AY739618) with reference sequence (Table 1).

Sequence comparisons identified a total of 48 and 8 variable sites for Cyt b and 12S rRNA respectively. This indicates Cyt b sequences of confiscated sample had higher variability with nearby species/subspecies comparison to 12S rRNA sequence. Appropriate Genetic DNA markers are those which are rather conservative within the species but discriminative between the species [44]. Cyt b shows limited variability within and much greater variation between species [45] thus ideal for species identification [42].

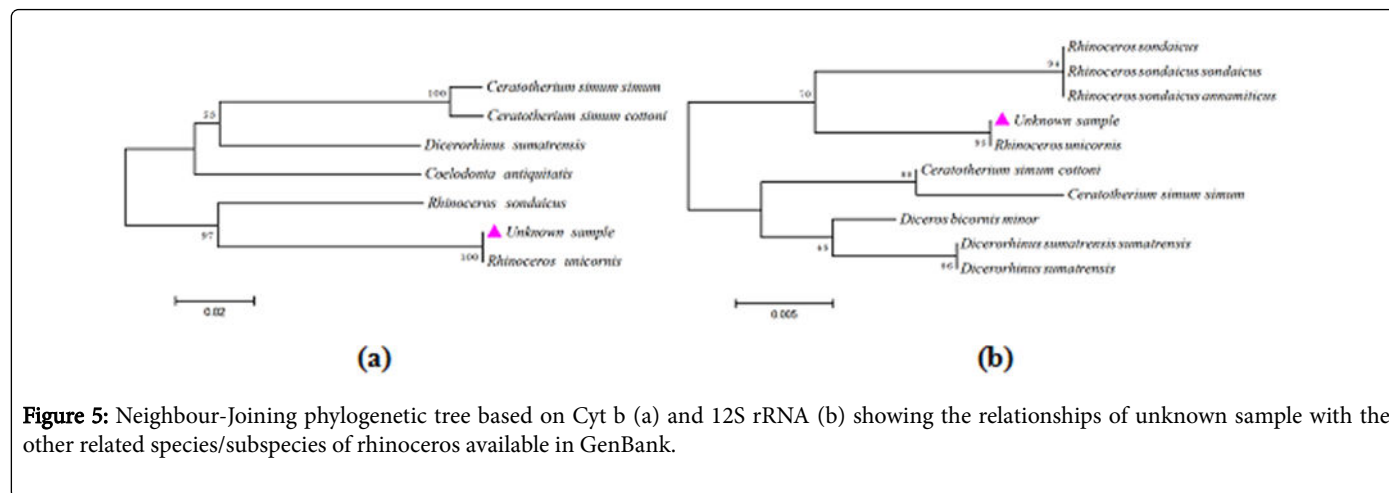


Figure 5: Neighbour-Joining phylogenetic tree based on Cyt b (a) and 12S rRNA (b) showing the relationships of unknown sample with the other related species/subspecies of rhinoceros available in GenBank.

The phylogenetic tree is used to discern the evolutionary relationships between the suspected sample and reference sequences from each possible species [5,46,47]. The position of the suspected sample in the tree allows the closest reference species to be identified as the likely source [7]. There are several different methods for constructing phylogenetic trees (e.g. neighbor-joining, maximum parsimony, maximum likelihood and Bayesian [7,48]). But, there is currently no consensus in wildlife forensic science over which to use [49,50]. The neighbor-joining (NJ) phylogenetic tree showed that BLAST analysis and neighbor-joining bootstrap values are similar in *Cyt b* gene. The NJ trees constructed using the sequences of *Cyt b* gene showed the similarity of confiscated sample to *Rhinoceros unicornis* with 100% bootstrap values (Figure 5a). The NJ tree made from sequences of *12S rRNA* gene also produced strong weight for the genetic similarity of suspected sample with *Rhinoceros unicornis* (Figure 5b).

Reference sequence database of different species those are highly in wildlife trades are available at Wildlife Institute of India [51] and such data was used to confirm the BLAST results. Sequences of the *12S rRNA* genes were aligned with the reference database of WII and constructed NJ trees showed the similarity of confiscated sample to *Rhinoceros unicornis* with 100% bootstrap values (Figure 6). This is required because The International Society of Forensic Genetics (ISFG) recommends the use of voucher specimen reference DNA sequence, an inhouse and authentic DNA database for species identification for forensic validation [52]. The most commonly used reference databases for comparative species identification searches are the NCBI/EMBL/ DDBJ database collaboration and BOLD [7]. Much of the initial sequence data held on BOLD are unauthenticated, limited regulation and erroneous sequences [53-56]. This can lead to false identifications if the target sample belongs to a previously uncharacterized species [57].

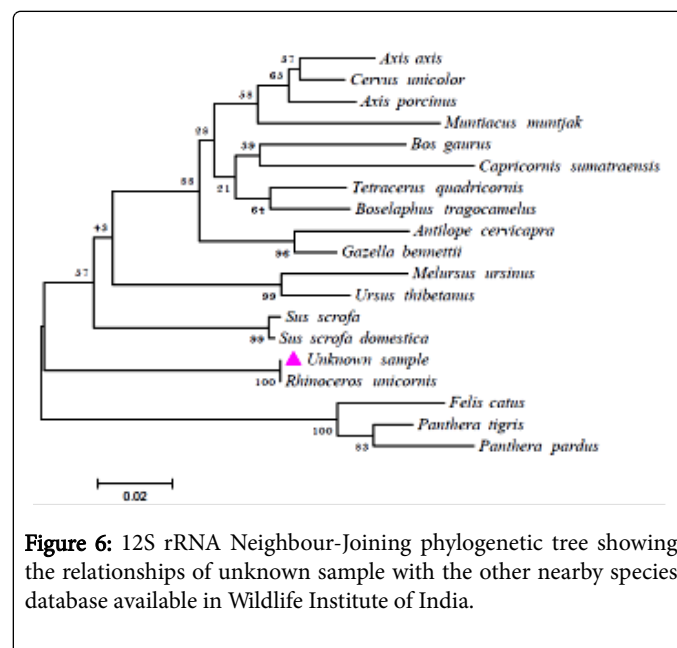


Figure 6: 12S rRNA Neighbour-Joining phylogenetic tree showing the relationships of unknown sample with the other nearby species database available in Wildlife Institute of India.

Conclusion

From the sequence alignment and a score table for the case sample based on Cyt b and 12S rRNA, the source of confiscated sample was found to *Rhinoceros unicornis*. DNA analysis cannot provide information like age [7] or body parts of the organism concerned. A hierarchy of techniques becomes more effective [58]. Thus, on the basis of genetic analysis result integrated with morphological and microscopic findings sample was ultimately confirmed as a nasal part

of infantile rhinoceros that bears horn. It is also concluded that infantile rhinoceros horn bears indistinct filamentous unit and laminae with wider filamentous unit space in comparison to mature horn. This is the primary and pioneer work which can be utilized by the scientific community for identification of infantile rhinoceros horn in future.

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