

## Original Research Article

# Scanning electron microscopy: a potential forensic tool to identify a piece of rhinoceros horn

Rina Rani Singh<sup>1\*</sup>, Naresh Kumar Saini<sup>2</sup>, Surendra Prakash Goyal<sup>3</sup>

<sup>1</sup>Department of Biotechnology and Biochemistry, Sardar Bhagwan Singh Post-Graduate Institute of Bio-medical Sciences and Research (S.B.S.P.G.I.), Post – Balawala, Dehradun–248161, Uttarakhand, India

<sup>2</sup>Retired from Wadia Institute of Himalayan Geology, 33, GMS Road, Dehradun -248001, Uttarakhand, India

<sup>3</sup>Retired from Wildlife Institute of India, Post Office - Chandrabani , Dehradun - 248001, Uttarakhand, India

**Received:** 28 December 2016

**Revised:** 10 January 2017

**Accepted:** 16 January 2017

### \*Correspondence:

Dr. Rina Rani Singh

E-mail: rina76india@yahoo.com

**Copyright:** © the author(s), publisher and licensee Medip Academy. This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial License, which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

### ABSTRACT

**Background:** Illegal trade of rhinoceros horn in the global market has posed a serious threat to the long-term survival and the conservation of endangered one horn rhinoceros. Presence of fake horns imitating rhinoceros horn makes the situation graver. Thus, to curb the trade it is very important to scientifically identify the horns used in trade to support and strengthen the enforcement agencies. The presence of fake items makes the identification task much more difficult thus, a project entitled “Characterization of species from bone, tusk, rhino horn and antler to deal wildlife offence cases” was developed to identify rhinoceros (*Rhinoceros unicornis*) horn in complete to pulverized form.

**Methods:** Various bio-scientific techniques like morphological, analytical and molecular were applied to identify the rhinoceros horn. Most of these techniques are promising in identifying rhinoceros horn in different forms. In present paper, we discuss on identification of a piece of rhinoceros horn using Scanning Electron Microscope and total nine horns were examined. The required micrographs were saved using software Digital Image Scanning System (DISS 5, Point Electronics, Germany). Scanned images for both rhinoceros horn from wild and zoo were similar. To find the specific signature, the micrographs of the rhinoceros horn were compared with buffalo and fake horns.

**Results:** Differences were noted on both dorsal and ventral surfaces of rhinoceros horns examined under scanning electron microscope. On ventral surface numerous uniformly placed circular pores with mean diameter of 320 µm and had characteristic “sub pores within a pore”. Instead, in fake horn only few non-uniform pores were visible without any sub pores. The ventral portion of buffalo horn does not indicate presence of any such pores.

**Conclusions:** The characteristic “sub pores within a pore” signature of the ventral portion of rhinoceros horn has high potential for forensic identification and consequently, proving offences and convicting offenders.

**Keywords:** Rhinoceros horn, Scanning electron microscopy, Forensic

### INTRODUCTION

There are five species of rhinoceros in the world, of which two, black rhinoceros (*Diceros bicornis*) and white rhinoceros (*Ceratotherium simum*) are found in Africa and rest three, Sumatran rhinoceros (*Didermoceros sumatrensis*), Javan rhinoceros (*Rhinoceros sondaicus*) and Indian rhinoceros (*Rhinoceros unicornis*) are found in Asia.<sup>1</sup> Both African species and one of the Asian

species (*Didermoceros sumatrensis*) has two horns, instead there is only one horn in other two Asian species.<sup>1</sup> All five species of rhinoceros are endangered and are placed in Appendix - I of the CITES.<sup>1</sup>

The rhinoceros populations in Africa and Asia have been badly affected due to reckless poaching and extensive loss of habitat.<sup>1-3</sup> the total population of Asian species is fewer than 2,700.<sup>4</sup> Presently, the Indian rhinoceros, which

is distributed in few pockets of India, Bhutan and Nepal, are estimated to be approximately 2000 out of which India has approximately 1500 Indian rhinoceros, confined to few areas of Assam (Kaziranga, Orang, Loakhawa & Pobitora), W. Bengal (Jaldapara & Gorumara) and Uttar Pradesh (Dudhwa).<sup>1,5-7</sup>

The prime cause of poaching is due to illegal trade of rhinoceros horns for its high price for traditional uses in medicines to cure fever, which does not have any other medicines, sculptors and dagger handles and thus highly prized.<sup>1,8,9</sup> Rhinoceros horn has been used as medicine in China, Burma, Thailand and Nepal in the treatment of haemorrhoids, arthritis, lumbago and polio.<sup>10</sup> Various published articles undertaken in Asia and Africa can illustrate the gravity of this trade.<sup>11,12</sup> As per a report 692 Great Indian one-horn rhinoceros were poached between 1980 and 1993 and another report reveals declined in rhinoceros population from 76 in 1966-67 to 14 by 1980 due to poaching in Jaldapara Wildlife Sanctuary, Assam, India.<sup>13,14</sup>

Steps have been taken to curb the reckless poaching. The international commercial trade of rhinoceros and their products was banned in 1976 under the CITES convention.<sup>2</sup> To reduce rhino horn demand its substitutes like horns of saiga antelope and water buffalo have also been tried.<sup>1,10,15</sup> Despite, the legal protections and prohibitory measures, illegal trade of rhinoceros horn and its products still continues, due to high demand in international market. Lack of proper identification methods for rhinoceros horn while, in processed and powdered form is further hindrance for enforcement agencies in convicting offenders.<sup>10</sup> Thus, characterization of the rhinoceros horn is very important to support legal intervention.

Therefore, we tried to develop characterization methods for identification of the Asian rhinoceros horn either complete or grounded using different techniques viz. Morphometry, Scanning Electron Microscopy (SEM), X-ray Diffraction (XRD), Elemental Analysis {X-ray Fluorescence (XRF), and Inductively Coupled Plasma – Mass Spectroscopy (ICP-MS)}, Thermo Gravimetric Analysis (TGA), and Molecular analysis. Attempts were made to develop species-specific characteristics of Asian rhinoceros horn (*Rhinoceros unicornis*) to differentiate from horn of other species. This will consequently help in reducing illegal trade of rhinoceros horn and will support in conservation of the endangered species.

Several studies have been undertaken to characterize rhinoceros horn of African species as well as Asian species based on various techniques.<sup>1,3,7,16,18</sup>

These studies reveal that the Rhinoceros horn is epidermal derivatives composed have keratinized tubules of cells embedded in keratinized amorphous matrix.<sup>19</sup> Reported that the tubules comprise of ~ 40 lamellae of epithelial cells and range from 300-500 µm in diameter.<sup>20</sup>

According to the amorphous matrix is made up of keratinized fusiform interstitial cells.<sup>21</sup> Several authors have used chemical techniques to differentiate species and geographical source of the African rhinoceros horn, based on the variation in the concentration of elements and isotopes and their ratios.<sup>3,22-25</sup> Investigated comparative biochemical composition of amino acids of keratins (eukeratins) of hair, wool, horn, nails, quills, feathers, skin and eggshell membrane.<sup>16</sup> He found the amount of cystine was comparatively low in rhinoceros horn, goat hair, cattle horn, porcupine quills and hen feathers. The tyrosine component was found highest (approximately 9%) in rhinoceros horn and echidna spines, as these are hard substance.

Molecular studies of rhinoceros tissue, horn and blood, mostly deals with phylogenetic issues.<sup>26-28</sup> whereas few authors have used molecular techniques to identify rhino horn, has also examined rhinoceros horn under scanning electron microscope, he has noted the dorsal surface structure of rhinoceros horn.<sup>7,8</sup> Studied the genetic characteristic of South African populations of black rhinoceros, and significantly differentiate between populations.<sup>29</sup> Report that *Rhinoceros unicornis* has 82 chromosomes, with male being heterogametic and female being homogametic.<sup>30</sup> Cloned and sequenced a 906 bp EcoRI repeat DNA fraction from *Rhinoceros unicornis* genome. He revealed that the AT rich contig pSS(R)2 sequences are unique to *Rhinoceros unicornis* genome because they do not cross-hybridize, even with the genomic DNA of South African black rhino *Diceros bicornis* and can be potentially used for identification of horn or other body tissues of *Rhinoceros unicornis*.<sup>7</sup> Developed a method to distinguishing between the African rhinoceros horns and those from various domestic and wild bovid species through extraction of DNA remaining in horn, amplification and direct sequencing of a portion of the mitochondrial Cytochrome *b* gene and comparing the sequence with a database of representative rhinoceros and bovid species.<sup>31</sup>

The above literature review shows that informative scientific studies have been done for identification of rhinoceros horn but most of them are on African species. Since, the Asian horns are believed to be more efficacious and comparatively expensive than African horns, the former are more prone for illegal trade. Almost all scientific studies are based on one or more than one techniques.<sup>1,32</sup> Therefore, it was intended to analyze the horn of Asian species and use most of the recent techniques available so, that wide spectrum of various forms of samples of rhinoceros horn can be identified. It will also help in differentiating it from fake horns, which are prevalent in trade.

## METHODS

All rhinoceros horns were procured from courtesy to Forest Departments namely, Assam, West Bengal and Uttar Pradesh and Delhi Zoo. Buffalo horn was procured

from Assam and Fake rhinoceros horns were seized and referred to Wildlife Institute of India for identification from U.P., Jharkhand and Sikkim. Based on the research work for characterization of rhinoceros horn, plan was to characterize the horn of great one horned rhinoceros using structural patterns, chemical constituents, protein

profile and DNA sequence. Table 1 indicates about the methods used and number of samples analyzed in the project. In present paper we deal with SEM technique only. This technique serves as a potential tool for forensic identification of horn of Great Indian Rhinoceros.

**Table 1: Different techniques used in characterizing rhinoceros and other horns.**

| Technique Applied                       | Rhinoceros horn |     |      | Buffalo horn |
|---|-----------------|-----|------|--------------|
|   | Wild            | Zoo | Fake |              |
| Morphometry                             | 6               | 2   | 2    |              |
| Scanning Electron Microscopy (SEM)      | 3               | 2   | 3    | 1            |
| X-ray Diffraction (XRD)                 | 4               | 1   | 2    | 1            |
| X-ray Fluorescence (XRF)                | 1               | -   | -    | -            |
| Inductively Coupled Plasma –MS (ICP-MS) | 2               | 2   | -    | -            |
| Thermo Gravimetric Analysis (TGA)       | 1               | 2   | -    | -            |
| Protein profile (SDS-PAGE)              | 3               | 2   | 1    | 1            |
| DNA Analysis                            | 2               | 1   | -    | 1            |

### Scanning electron microscopy

Total nine horns (three rhinoceros horns from wild, two from zoo, three fake rhinoceros horns and one buffalo horn from wild) were examined under scanning electron microscope as shown in Figure 1.



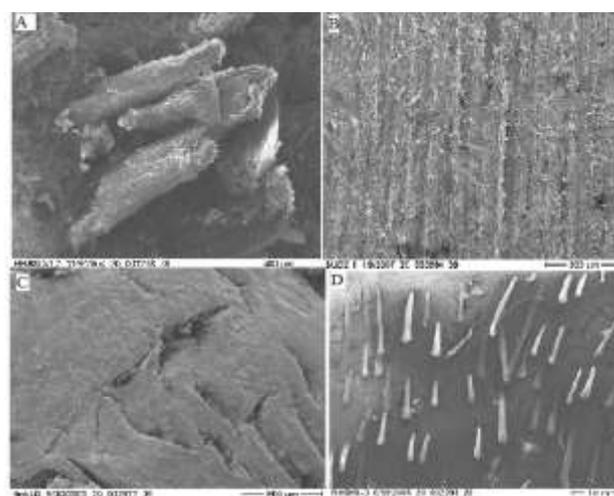
**Figure 1: Samples used in the study (A. Rhinoceros horns from wild; B. from zoo; C. Fake rhinoceros horns and D. Buffalo horn).**

Samples were prepared by cutting two small pieces of 5 mm width, 5 mm length and 2 mm thickness, one from dorsal and other from ventral side from basal portion of rhinoceros horn and cleaned properly using alcohol. These small pieces were mounted on the stub using silver adhesive paste and left for drying. The dried samples were sputter coated with gold (thickness 10-20 Å) using ion beam current of 25 mA at 2.5 kV in argon atmosphere at 10-8 mbar/pa for 3 minutes using cool sputter coater (E5100 Series II, Polaron Equipment Ltd., U.K.) to facilitate their examination under the Scanning Microscope (PSEM 515, Philips, Holland). The required

micrographs were saved using software digital image scanning system (DISS 5, Point Electronics, Germany). The micrographs obtained of the rhinoceros horns and fake rhinoceros horns were compared on both dorsal and ventral side, to note the species-specific characteristics and differentiate them.

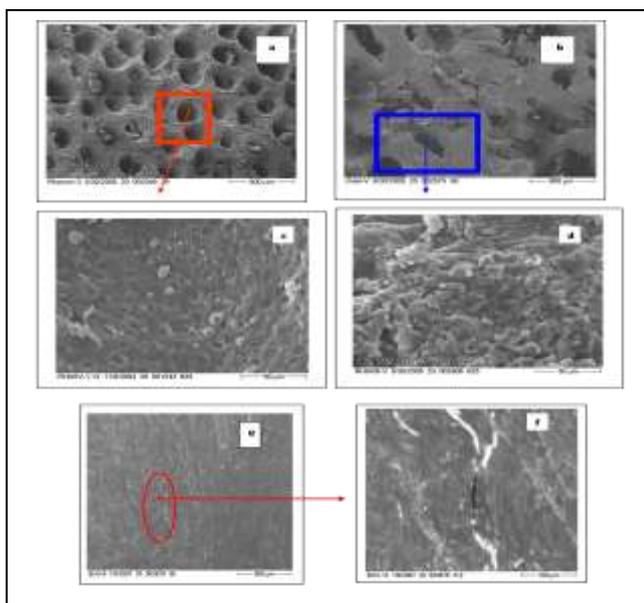
### RESULTS

Differences were noted on both dorsal and ventral surfaces of rhinoceros horns examined under scanning electron microscope. On the dorsal surface hairs were observed growing compactly and profusely in real rhinoceros horn as shown in Figure 2A, instead in buffalo and fake horn there were no evidence of hairs as given in Figure 2B and 2C, except in a sample where sparse hair growth were observed as presented in Figure 2D. In rhinoceros horn from zoo, hairs were not noted.



**Figure 2: Scan micrographs of dorsal portion of A. Rhinoceros horn; B. Buffalo horn; C. Fake rhinoceros horn and D. Fake rhinoceros horn with planted hairs.**

On examination of ventral surface numerous uniformly placed circular pores with mean diameter of 320  $\mu\text{m}$ , which range from 160-400  $\mu\text{m}$  were noticed as shown in Figure 3a. When a pore was magnified further, many sub-pores of mean diameter of 9.39  $\mu\text{m}$ , which range from 5.56 to 10.11  $\mu\text{m}$  were visible within that pore as shown in Figure 3c. Instead, in fake horn only few non-uniform pores were visible (Fig. 3b and on its further magnification no sub-pores were visible but rough surface were visible as shown in Figure 3d). The ventral portion of buffalo horn does not indicate presence of any such pores as shown in Figure 3e its further magnification reveals presence of twisted fibers, which had dark zone in the middle of the twisted fibers as shown in Figure 3f.



**Figure 3: Scan micrographs of ventral portion of horn. a. Rhinoceros; b. Fake; c. Magnified micrographs of ventral portion of Rhinoceros horn; d. Magnified micrographs of fake horn; e. Scan micrograph of buffalo horn; f. Magnified micrographs of buffalo horn.**

## DISCUSSION

Although, Morphometry is one of the cheap and best methods to identify an object when it is intact and can be done at the field itself and by ground level staff. Various scientists have studied Morphometry of rhinoceros horns of various species.<sup>1,17,18,33</sup>

When finished product of rhinoceros horn is seized it is sometime difficult to characterize the rhinoceros horn through morphometry. Has done an excellent study based on microscopy and x-ray tomography, his study deals with ultrastructure of the horn under microscope and ultraviolet light.<sup>17</sup> Few studies are also reported using scanning electron micrography of rhinoceros horn.<sup>21,34</sup> Identified sculptors made up of rhinoceros horn using

SEM and microscopic characteristics to use it for molecular study.<sup>8</sup>

In present study scanning electron microscopy was used to study both dorsal and ventral surface and the structure noticed were distinct that is presence of compact hair growth dorsally as seen in Figure 2a and presence of uniformly placed pores having mean diameter of 320  $\mu\text{m}$  and these range from 160 to 400  $\mu\text{m}$  on the ventral surface as shown in Figure 3a. Hair like structure was also noticed by.<sup>34</sup> Presence of sub-pores having diameter 9.39  $\mu\text{m}$  and these ranges from 5.56 to 10.11  $\mu\text{m}$  within a pore is very remarkable and can be used for identification of rhinoceros horn as seen in Figure 3c. These pores seem to be similar to the tubules examined by Hieronymus et al. The pattern of “sub-pores within a pore” is unique to rhinoceros horn and it is very difficult to imitate it with uniformity thus, it can be important tool for forensic identification.

We also attempted to identify three suspected rhinoceros horn seized in offences using SEM and other techniques and could successfully differentiate them based on above unique surface morphology of rhinoceros horn, these samples were found to be fake. The buffalo horn micrographs of dorsal surface does neither indicates presence of hairs as shown in Figure 2b nor pores were present on the ventral surface instead there were presence of twisted fibers which had dark zone in the center as seen in Figure 3e and 3f thus can be differentiated.

Thus, SEM technique used indicates that great Indian rhinoceros horn has unique signatures. These unique signatures can serve as tools to identify intact and pieces rhinoceros horn, and sculptors, contributing in conservation of great one horn rhinoceros species. SEM may be used to characterize other rhinoceros horns available globally to find differences between horns obtained from different species. Other techniques like X-ray diffraction, Thermo gravimetric analysis, protein profile and DNA are useful in identification of pulverized rhinoceros horn. SEM technique has potential for forensic identification of rhinoceros (*Rhinoceros unicornis*) horn.

## ACKNOWLEDGEMENTS

We are thankful to the Director and Dean, Wildlife Institute of India for the support for research and development work. We are grateful to the Director, Delhi Zoo and the Chief Wildlife Wardens of Uttar Pradesh, West Bengal, and Assam for providing samples for research purpose. Our sincere thanks are due to the Director, Wadia Institute of Himalayan Geology (WIHG), Dehradun and Scientists namely, Dr. P. K. Mukherjee, and Dr. P. P. Khanna for their help while using their instrumental facilities. Thanks are due to Mr. N. K. Juyal, technician for his contribution in SEM analysis. Sincere thanks are due to Faculty, Staff, and Friends at Wildlife Institute of India for their direct and indirect help. Thanks to Laboratory Assistant Mr. Sanjay

Chouniyal for his sincere hard work in sample preparation. We are thankful to our colleague in the laboratory for encouragement and our families for their patience and encouragements.

*Funding: The study was funded from Grant-in-aid from Ministry of Environment and Forests, Lodi Road, New Delhi, India*

*Conflict of interest: None declared*

*Ethical approval: Not required*

## REFERENCES

- Dinerstein E. The return of the unicorn. In: the natural history and conservation of the greater one-horned rhinoceros. New York: Columbia University Press; 2003.
- Leader-Williams N. The World trade in Rhino horn: A review. Traffic International. 1992: 40.
- Amin R, Bramer M, Emsile R. Intelligent data analysis for conservation: experiments with rhino horn fingerprint identification. Knowledge-Based Sys. 2003;16:329-36.
- Foose TJ, van Strien N. Asian Rhinos. Status Survey and Conservation Action Plan. IUCN/SSC Asian Rhino Specialist Group. Switzerland: IUCN, Gland,; 1997
- Khan BMK, Foose TJ. Chairman's Report: Asian Rhino Specialist Group. Pachyderm. 1994;18:3-8.
- Hanfee F. Wildlife Trade: A Handbook for Enforcement Staff. WWF-Tiger Conservation Program 1998: 56.
- Ali S, Azfer MA, Bashamboo A, Mathur PK, Malik PK, Mathur VB et al. Characterization of a species-species repetitive DNA from a highly endangered wild animal, Rhinoceros unicornis, and assessment of genetic polymorphism by microsatellite associated sequence amplification (MASA). Gene. 1999;228:33-42.
- Hsieh HM, Huang LH, Tsai LC, Kuo YC, Meng HH, Linacre A, Lee JCI. Species identification of rhinoceros horns using the cytochrome b gene. Forensic Sci Int. 2003;136:1-11.
- Emslie RH, Brooks PM. African rhino. In: status survey and conservation action plan IUCN/SSC African Rhino Specialist Group. UK: IUCN, Gland, Switzerland and Cambridge; 1999.
- Penny M. Rhinos: Endangered Species. London: Christopher Helm Publishers Limited;1987.
- Martin EB, Vigne L, Allan C. On a knife's edge: The rhinoceros horn trade in Yemen. Cambridge: Traffic International; 1997.
- Sherriffs P. Black rhino released onto Zululand Rhino Reserve. Pachyderm. 2005;39:113.
- Menon V. Rhino horn trade: Threats of rhino horn trade to the species in India. New Delhi. CBI Bulletin. 1996;4(2):29-33.
- Mukherjee S, Sengupta S. Rhino poaching in Jaldapara Wildlife Sanctuary, North Bengal, India. Tiger paper. 1998;25(1):20-1.
- Hay PP. Ethnopharmacology of rhinoceros horn. II: antipyretic effects of prescriptions containing rhinoceros horn or water buffalo horn. J Ethnopharmacol. 1991;33(1-2):45-50.
- Block RJ. The composition of keratins: The amino acid composition of hair, wool, horn, and other eukeratins. J Biol Chem. 1939;127:181-6.
- Hieronimus TL, Witmer LM, Ridgely RC. Structure of white rhinoceros (*Ceratotherium simum*) horn investigated by X-ray computed tomography and histology with implications for growth and external form. J Morpol. 2006;267:1172-6.
- Kingdon J. East African mammals. Volume 3B. Chicago: University of Chicago Press; 1979
- Hieronimus TL, Witmer LM. Rhinoceros horn attachment: anatomy and histology of a dermally influenced bone rugosity. J Morphol. 2004;260:298.
- Ryder ML. Structure of rhinoceros horn. Nature. 1962;193:1199-201.
- Lynch LJ, Robinson V, Anderson CA. A scanning electron microscope study of the morphology of rhinoceros horn. Aust J Biol Sci. 1973;26:395-9.
- Lee-Thorp JA, van der Merwe NJ, Armstrong RA. Final Project Report ZA309: Source area determination of rhino horn by isotopic analysis. Unpublished WWF Report; 1992.
- Hall-Martin AJ, van der Merwe NJ, Lee-Thorp JA, Armstrong RA, Mehl CH, Struben S, Tykot R. Determination of species and geographic origin of rhinoceros by isotopic analysis and its possible implication to trade controls. San Diego, California: Proceedings of an International Rhino Conference; 1993: 123-124.
- Hart RJ, Tredoux M, Damarupurshad A. The characterization of rhino horn and elephant ivory using the technique of neutron activation analysis. South Africa: Final report on a project undertaken on behalf of the Department of Environmental Affairs; 1994.
- Emslie RH, Brooks PM, Lee-Thorp JA, Jolles A, Smith W, Vermaas N. Development of a continental African rhino horn fingerprinting database and statistical models to determine the probable species and source of rhino horn. AFRSG rhino horn fingerprinting for security project 9F0084.1. Unpublished report to WWF; 2001.
- Ashley MV, Melnick DJ, Western D. Conservation genetics of the black rhinoceros *Diceros bicornis*, I: evidence from the mt-DNA of three populations. Conserv Biol. 1990;1:71-7.
- Swart MKJ, Ferguson JWH, Toit R du, Flamand JRB. Substantial genetic variation in the African black rhinoceros (*Diceros bicornis*). J Heredity. 1994;85:261-6.
- Tougard C, Delefosse T, Hanni C, Montgelard C. Phylogenetic relationship of the extant rhinoceros species (*Rhinocerotidae*, *Perissodactyla*) based on mitochondrial cytochrome b and 12 S rRNA genes. Mol Phylog Evol. 2001;19(1):34-44.

29. Swart MKJ, Ferguson JWH. Conservation implication of Genetic differentiation Southern African populations of black rhinoceros (*Diceros bicornis*). *Conservation Biol.* 1997;11:79-83.
30. Wurster DH, Benirschke K. The chromosomes of the great Indian rhinoceros (*Rhinoceros unicornis* L.). *Experientia.* 1968;24:5-11.
31. Hoesch RM, Steven RF. Species identification of mammalian horn by PCR. Ashland, Oregon, USA: National Fish and Wildlife Forensics Laboratory; 2004.
32. Nowell K, Chyi WWL, Pei KCJ. The horns of a dilemma: the market for rhino horn in Taiwan. Cambridge, United Kingdom: Traffic International; 1992: 44.
33. Groves CP. Species characters in rhinoceros horns. *Zeitschrift fur Saugetierkunde.* 1971;36:238-52.
34. Hsieh HM, Chiange HL, Tsai LC, Lai SY, Huang NE, Linacre A, et al. Cytochrome b gene for species identification of the conservation animals. *Forensic Sci Int.* 2001;122:7-18.

**Cite this article as:** Singh RR, Saini NK, Goyal SP. Scanning electron microscopy: a potential forensic tool to identify a piece of rhinoceros horn. *Int J Sci Rep* 2017;3(2):33-8.