Science & Justice 59 (2019) 622–629

Contents lists available at ScienceDirect

Science & Justice

journal homepage: www.elsevier.com/locate/scijus

Rapid and non-destructive identification of claws using ATR-FTIR spectroscopy–A novel approach in wildlife forensics

Chandra Prakash Sharma[a], Sweety Sharma[b], Vishal Sharma[c], Rajinder Singh[b,*]

[a] Wildlife Forensic cell, Wildlife Institute of India, Dehradun, Uttarakhand, India
[b] Department of Forensic Science, Punjabi University, Patiala, Punjab, India
[c] Institute of Forensic Science and Criminology, Panjab University, Chandigarh, India

ARTICLE INFO

Keywords:
Wildlife forensics
Wildlife trafficking
Claws
ATR-FTIR spectroscopy
Chemometrics

ABSTRACT

Differentiation and identification of Royal Bengal Tiger (*Panthera tigris tigris*) and Indian Leopard (*Panthera pardus fusca*) claws is a challenging task in wildlife forensics, due to similarity in their morphology, anatomy and chemical compositions as both the species are closely related to each other genetically. ATR-FTIR spectroscopy, which offers a non-destructive and safe alternative technique to other conventional methods, has been employed in the present work to differentiate claws of Royal Bengal Tiger and Indian Leopard. An attempt has been made to differentiate 31 reference claw samples from 16 different Royal Bengal Tigers, 15 different Indian Leopards, and 10 fake claws using ATR-FTIR spectroscopy supplemented with PCA, PLS-DA, and LDA. PCA could not distinguish the samples of two closely related species among themselves as well as from the fake claws. On the other hand, PLS-DA and LDA models both yielded highly significant classification rate for differentiation among the samples of Royal Bengal Tiger, Indian Leopard, and their fake counterparts. Further, seven blind claw samples that were pretended to be unknown to the analyst of both the species are also examined and identified correctly to their respective groups. The R-Square value obtained for PLS-DA model to differentiate Royal Bengal Tiger, Indian Leopard, and fake claws is 0.99, which is highly significant for predictive accuracy. This study shows that ATR-FTIR spectroscopy with PLS-DA/LDA has a potential to present a rapid, non-destructive, reliable, and eco-friendly approach for the accurate identification and differentiation of Royal Bengal Tiger and Indian Leopard claws.

1. Introduction

Wildlife crime is posing a serious threat to biodiversity and environment. A huge amount of money, approximately $ 32 billion involved in the illegal trade of wild animals, their bodily parts and products thereof is raising serious issues with internal security of many nations across the globe as well [1–3].

Every year, wild animals in large numbers are poached to fulfill crazy and money-grubbing desires of human being. The incident of poaching of Royal Bengal Tiger (*Panthera tigris tigris*) and Indian Leopard (*Panthera pardus fusca*) among all other wild cats are more frequent due to the heavy demand of their body parts and products in this trade [4,5]. In India, both species are declared critically endangered and are protected under schedule 1 of the Wildlife (Protection) Act-1972. At the international level, both are listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), which prohibits international commercial trade of these species, their body parts or other derivatives between all CITES Parties. India has been a signatory to CITES since 1976 [6].

Along with skin and bones, claws are the most sought-after articles in the market and constitute to 3.8% of total articles (n = 2899) related to wildlife crime received at Wildlife Institute of India, Dehradun for forensic examination [7]. Claws are used to make pendants, sculptures, items of religious worship and Traditional Chinese Medicines (TCMs) etc. High demand for Royal Bengal Tiger and Indian Leopard claws has caused the heavy influx of fake claw articles in this trade as well [7]. Due to the extensive or wide range of similarity in appearance and morphology of Royal Bengal Tiger and Indian Leopard claws and their fake counterparts, it is of paramount importance to differentiate and identify them for the successful implementation of laws related to wildlife protection and conservation.

For the analysis of claws various techniques such as of X-ray analysis (Radiography), Burn test, morphological and DNA-based techniques have been used in past [7]. X-ray analysis and morphological

* Corresponding author at: Department of Forensic Science, Punjabi University, Patiala, Punjab 147002, India.
E-mail address: rajchandel7@gmail.com (R. Singh).

https://doi.org/10.1016/j.scijus.2019.08.002
Received 28 March 2019; Received in revised form 24 July 2019; Accepted 11 August 2019

examination, which require thorough knowledge of claw morphology and anatomy is rapid and non-destructive in nature however cannot be applied to the samples, which are fragmented/mutilated/modified. The Burn test (a keratin-specific pungent smell is produced, when a small piece of claw is burnt in a flame, due to the formation of sulfur compounds) is both destructive and subjective. DNA based species identification is the most commonly employed and widely accepted method to identify claws. However, DNA analysis from keratinized materials is a laborious and costly process, which involves usage of harmful chemicals and destructive as well [8].

Recently, spectroscopic techniques have emerged as a tool for species identification from a variety of biological materials. Human, cat and dog blood was successfully differentiated using Raman and ATR-FTIR spectroscopy coupled with principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) respectively [9,10]. Human and animal blood was successfully differentiated using Raman spectroscopy [11–14], Diffuse reflectance spectroscopy [15,16], Near-infrared diffuse transmittance spectroscopy combined with PLS-DA method [17,18] and ATR-FTIR spectroscopy [19]. Pickering et al. [20] reported the discrimination of maggot's species and its life cycle stages simultaneously by using ATR-FTIR spectroscopy in combination of principal component- discriminant function analysis (PC-DFA), and support vector machine (SVM). In addition, human, cats, dog hair [8], human and non-human bone [21] were also successfully differentiated by using ATR- FTIR spectroscopy. The application of FTIR for non-destructive differentiation of hard tissues of animals especially in members of closely linked families is still in its infancy stages. However, the following works can be considered as a stepping stone to move forward. Espinoza et al. [22], Guo et al. [23], Walker, [24], and Nagaraju [25] have worked on the identification of Elephants and Giraffe hair, straight guard hair of Golden cat and Indian Leopard cat, various animal hard tissues, and skin and appendages of wild animals respectively. Therefore, this study is a step further in this direction.

In this pilot study, ATR-FTIR spectroscopy supplemented with chemometrics is used to differentiate and identify claws of Royal Bengal Tigers, and Indian Leopards with external and blind validation testing. As per the authors' best of knowledge, no study has been reported for the purpose of species identification from claws using ATR-FTIR spectroscopy till date. From a forensic perspective, this technique could be an ideal tool because of its sensitivity, rapid analysis, eco-friendly, and non-destructive nature with a high degree of confidence and minimal to no sample preparation requirements. The main purpose of utilizing the chemometric methods for such kind of problems is that the spectroscopic analytical methods (such as FTIR in the present study) generate a huge amount of dataset creating difficulties in the interpretation of the results from these dataset. This kind of problems could potentially be overcome through the use of chemometric techniques. The data/spectrum obtained through ATR-FTIR analytical method varies from sample to sample and these chemometric methods extract different useful information for the purpose of classification and prediction. The individualization and classification in a particular class of samples is known as 'chemical pattern recognition' [26]. These pattern recognition methods are having two types namely supervised and unsupervised pattern recognition. PLS-DA and LDA which come under supervised pattern recognition have been utilized for classification purpose here.

2. Materials and methods

2.1. Sample collection details

A total 31 Claw samples from 16 different Royal Bengal Tigers (*Panthera tigris tigris*) and 15 different Indian Leopards (*Panthera pardus fusca*) as training data set were obtained from the repository of Wildlife Institute of India (WII), Dehradun. Additionally, a test dataset of 10 claw samples, proven fake (with the help of X-ray, morphometric measurements, and burn test) was prepared for external validation test to assess the performance of classification model. The fake samples were made up from keratinized materials most likely hooves and horn of other species. A set of seven samples (41, 53, 54, 81, 82, 163, and 164) was also prepared for the blind test. These samples were pretended to be unknown for the analyst and their actual identity was not revealed until predictions were completed. These samples were not the part of training data set.

2.2. Sample preparation

Fine scrapings across the claws samples were obtained by using surgical blades (Pulse™). The scrapings were placed on the crystal surface and analyzed directly without giving any pre-treatment to the samples.

2.3. Sample analysis

A Bruker Alpha Fourier Transform Infrared (FT-IR) spectrometer with a Smart Orbit; ZnSe crystal attenuated total reflectance (ATR) accessory and OPUS (V 7.2) software equipped with an air-cooled DTGS detector was used to collect the spectra of all samples. Acetone was used throughout the experiments to clean the ATR stage supplied by Loba Chemie Company to avoid any cross contamination. The spectra were measured from 4000 to 600 cm^{-1} with a resolution of 4 cm^{-1}. The signal was averaged from 23 scans. The small amount of sample was put directly on a ZnSe crystal and pressed carefully with an ATR pressure anvil. Background was analyzed each time before the analysis. Principal component analysis (PCA) and Partial least square- discriminant analysis (PLS-DA) were applied using the trial version of Unscrambler X (CAMO Software AS, Oslo, Norway) software on the infrared spectroscopic data. Data were imported in opus format in the whole mid infrared spectral range (4000–600 cm^{-1}) in Unscrambler X software. The LDA analysis was performed with SPSS software (20.0 IBM). The result analysis and the graphical plots were performed using the Origin Pro 8 (Origin Lab Corporation, Massachusetts, USA) and SPSS software (20.0 IBM).

2.4. Chemometric methods

Chemometric is the application of mathematical and statistical methods to design, extract, and interpret the information from the large dataset obtained from various analytical techniques [27].

2.4.1. Principal component analysis

PCA is an unsupervised dimensionality-reduction technique which reduces large data set to few significant variables or co-ordinates called principal components for easy pattern recognition and relationships in data. It is used to reduce multicollinearity and explains the variance in a data set without any loss of information. PCA results are commonly displayed in the form of score plots that represent the similarities and differences between the data sets that helps in easy interpretation of the original data. In score plots, sample with similar score forms a group and samples with different score are placed distant from one another [27–29].

2.4.2. Partial least square-discriminant analysis (PLS-DA)

Partial least square-discriminant analysis (PLS-DA) has evolved from PLSR algorithm which combines features of both PLS (determination of suitable variables) and discriminant analysis (classification of samples based on the extracted variables). PLS-DA is a supervised linear classification chemometric technique exhibiting the properties of PLSR with the discrimination power of predictive and classification ability with the ability to handle correlated and multicollinear variables in the data [30,31].

Firstly, the generated classification model is calibrated, and then unknown samples are predicted in the pre-defined classes. PLS-DA

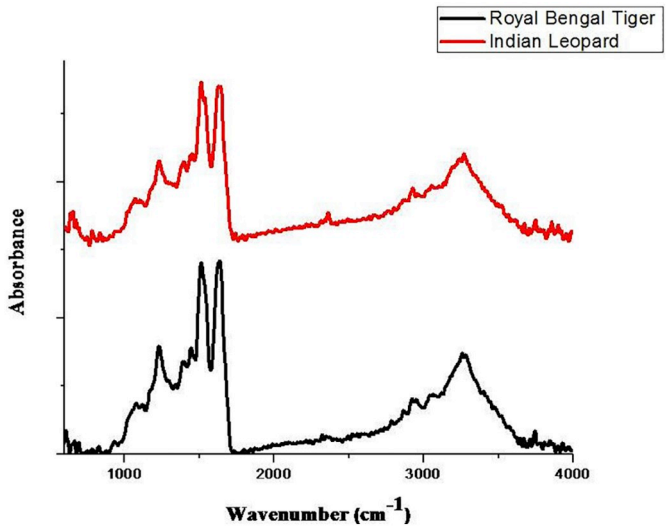

Fig. 1. Overlaid ATR-FTIR spectrum of Royal Bengal Tiger, and Indian Leopard in 4000–600 cm^{-1} wavenumber range.

searches for the latent variables with highest covariance with the Y-variables. It uses dummy values for the Y matrix as in if the value of predicted class is less than or more the value of threshold indicates that the sample belongs to other class [21,32].

The raw datasets obtained from ATR-FTIR spectroscopy were processed using PLS-DA analysis to identify the significant differences and similarities within score plots. The plot can be used to interpret differences and similarities among samples and samples within clusters are similar to each other. The pre-treatment applied for PLS-DA model was baseline offset and linear baseline correction, de-resolve transform and orthogonal signal correction. The algorithm used in the PLS-DA model was non- linear iterative partial least squares (NIPALS) with random cross validation method. This algorithm handles missing values and tends to be faster than Kernal-based algorithms.

2.4.3. Linear discriminant analysis (LDA)

LDA is the method of constructing linear combinations of canonical variables with minimum to no loss in the original information present in the datasets. This is done in order to maximize the separation among the already existing classes. LDA helps to build a model of prediction that allows the grouping of the unknown sample to the known classes based on the similarities and dissimilarities amongst them [28].

3. Results and discussion

Before analyzing any sample, the important experimental parameters were appropriately optimized. It is well understood that when the number of scans are increased, it will give a spectrum with good SNR (signal to noise ratio) and therefore improvement in the spectral quality. The resolution of spectrometer measures the ability of the instrument that how well it will separate two or more closer peaks. The spectra were measured from 4000 to 600 cm^{-1} with a resolution of 4 cm^{-1}.The signal was averaged from 23 scans. The obtained spectra are interpreted by peak comparison/visual inspection, PLS-DA and LDA chemometric methods.

3.1. ATR-FTIR spectrum of Royal Bengal Tiger and Indian Leopard claws

Claws are composed of keratin proteins, the significant peaks were observed in the fingerprint region ranges from 1600 to 600 cm^{-1} as shown in Fig. 1.

This region contains the major amide bands. The major peaks are located at approximately 3271 cm^{-1} [Amide A]; 1645 cm^{-1} [C═O stretching (Amide I)], 1517 cm^{-1} [CN stretching, NH bending (Amide II)], 1116 cm^{-1} (C—H stretching), 1454 cm^{-1} (asymmetric CH_3 bending vibration), 1396 cm^{-1} (symmetric CH_3 bending, aliphatic side groups of amino acid residues), 1233 cm^{-1} [CN stretching and NH bending vibrations (Amide III)], 702 cm^{-1} [out of plane N—H bending vibrations (Amide IV)], and 1080 cm^{-1} [cysteine oxidase (S—O) and cysteic acid] [25,33–39].

As shown in Fig. 1, ATR-FTIR spectra of Royal Bengal Tiger and Indian Leopard claws are almost similar and exhibit nearly identical values of absorbance and wavenumbers. It is difficult to differentiate the claws of Royal Bengal Tiger and Indian Leopard on the basis of their visual inspection.

3.2. Discrimination using PCA model

As shown in Figs. 2 and 3, using PCA the samples could not be differentiated into separate clusters of Royal Bengal Tiger and Indian Leopard class (Fig. 2), and class of Royal Bengal Tiger, Indian Leopard and fake claws (Fig. 3) and show scattered distribution which results in overlapping of Royal Bengal Tiger and Indian Leopard claw spectra. PC1 accounted for 80% of total variation, PC2 accounted for 8% of the variation, and PC3 summarized 4% of the remaining variation. Total 92% of the variation was observed with PC1, PC2, and PC3. Three-dimensional PCA scatter plot was generated which is shown in Fig. 2.

For the discrimination for Genuine Royal Bengal Tiger and Indian Leopard claws from their fake counterparts, PC1 accounted for 63%, PC2 accounted for 16%, and PC3 showed 4% of total variation. Cumulative variance of PC1, PC2, and PC3 was obtained to be of 83%. It was also observed that only 70% of fake claws (7 out of 10 samples) were accurately classified as a distinct cluster and henceforth, could be differentiated from their real counterparts (Fig. 3).

3.3. Discrimination between Royal Bengal Tiger and Indian Leopard claws using PLS-DA model

In an attempt to get better classification rate PLS-DA is applied. Two dimensional PLS-DA model is constructed using training dataset of 31 Infrared spectra of Royal Bengal Tiger and Indian Leopard claws with two specified latent variables or factors. The primary aim of the study is to differentiate Royal Bengal Tiger and Indian Leopard claws using FT-IR spectroscopy and chemometric methods. Based on PLS-DA score plot, Royal Bengal Tiger and Indian Leopard claws entirely separated from each other along with factor 1, which explains most of the variability as shown in Fig. 4. All the samples of Royal Bengal Tiger and Indian Leopard claws are categorized in their respective classes. No false negative and positive assignments were observed in this study and hence achieved 100% accuracy in terms of positive classification. The R-Square value obtained for this model is 0.999, which is highly significant. High R-square value denotes low error rate and high predictive accuracy. Thus the generated model is a good fit for the samples of the present study.

3.3.1. External validation test

For the external validation test, 10 fake Royal Bengal Tiger and Indian Leopard claws (not included in original training data set) were acquired. The samples were analyzed under similar set of experimental conditions. The samples were loaded in the PLS-DA model for the predictions. The results obtained with PLS-DA score plot are shown in Fig. 5.

From the score plot (Fig. 5), it is evident that complete differentiation of selected fake and genuine claws is achieved. The results demonstrate that, it would be very difficult for fake claws to be misidentified as a genuine Royal Bengal Tiger and Indian Leopard claws. This remarkable performance of the classification ability of PLS-DA model and the non-destructive approach of ATR-FTIR spectroscopy make this technique well suited for the forensic species discrimination.

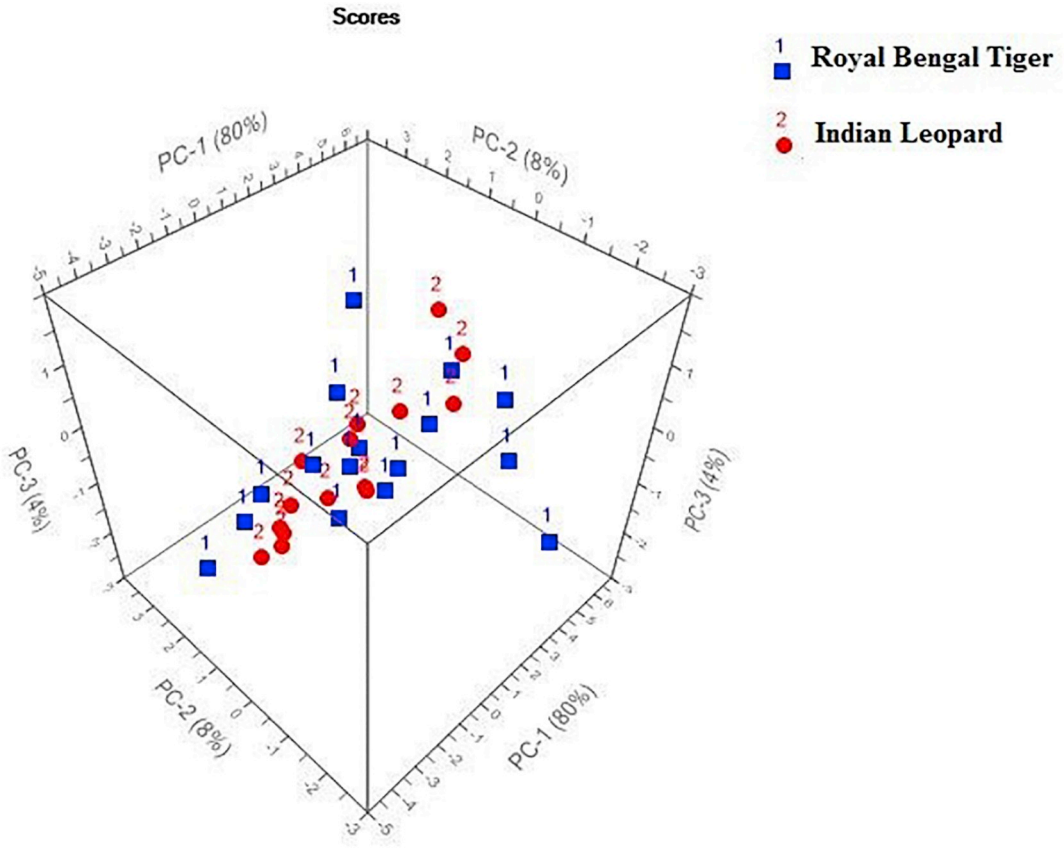

Fig. 2. PCA score plot to discriminate samples of Royal Bengal Tiger and Indian Leopard claws.

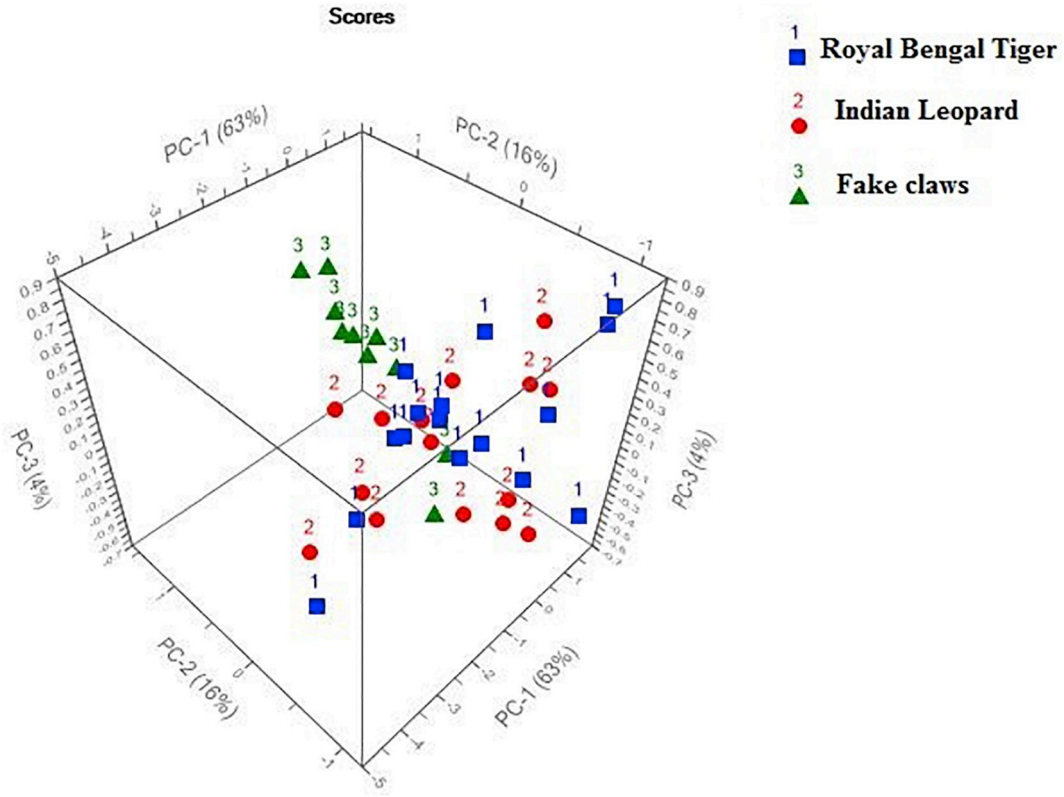

Fig. 3. PCA score plot to discriminate samples of Royal Bengal Tiger, Indian Leopard, and Fake claws.

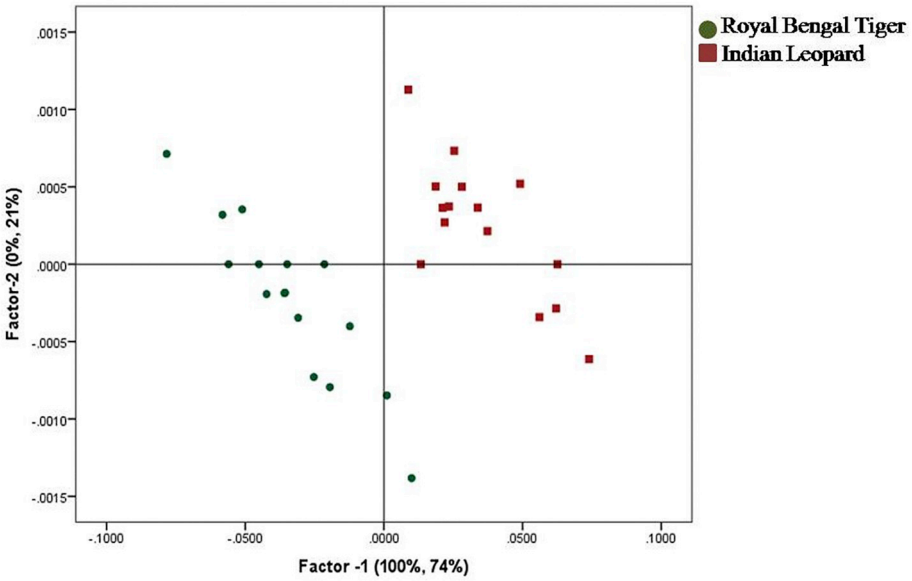

Fig. 4. Scatter plot to differentiate between Royal Bengal Tiger and Indian Leopard claws.

The results suggested that the complete differentiation is possible between fake and genuine claws with PLS-DAscore plot. The R-Square value obtained for this model is 0.998.

3.3.2. Blind test

To confirm the integrity of model that is the classification ability of this model, a blind validation test is performed. Seven unknown Royal Bengal Tiger and Indian Leopard claw samples were obtained (not included in training dataset). These samples were pretended to be unknown to the analyst and their actual identity was not revealed until predictions were completed.

The spectra of these samples are loaded into the PLS-DA model. Unknown samples can be identified by its nearest position of known group (Royal Bengal Tiger-1, Indian Leopard-2), which were made on the basis of training data set. In prediction columns, it is observed that all the unknown samples were positioned correctly in their respective class. The prediction results for these samples are displayed in Figs. 6, 7 and Table 1. In Fig. 6, marks with blue color are the predicted class for unknown samples. In Fig. 7, predicted Y represents groups of known class (1- Royal Bengal Tiger, 2- Indian Leopard, 3and 4-Unassigned) and X-axis represents samples of unknown claws. Sample number 163, 164, 53, and 54 lie in group 1 that is Royal Bengal Tiger class and sample number 41, 81, and 82 lie in group 2 that is Indian Leopard class. (see Table 2.)

These results demonstrate the model's ability to assign all the unknown samples to their respective class correctly that is Royal Bengal Tiger (Class1) and Indian Leopard (Class 2). The model resulted in 100% accuracy for the unknown claws sample's prediction, as none of samples is misclassified.

3.4. Linear discriminant analysis (LDA)

Although the PLS-DAmodel showed very good results in order to achieve the objective of the study. However one limitation in PLS-DA model is that the statistical software is needed to be run every time for predicting the unknown samples to establish whether they belong to

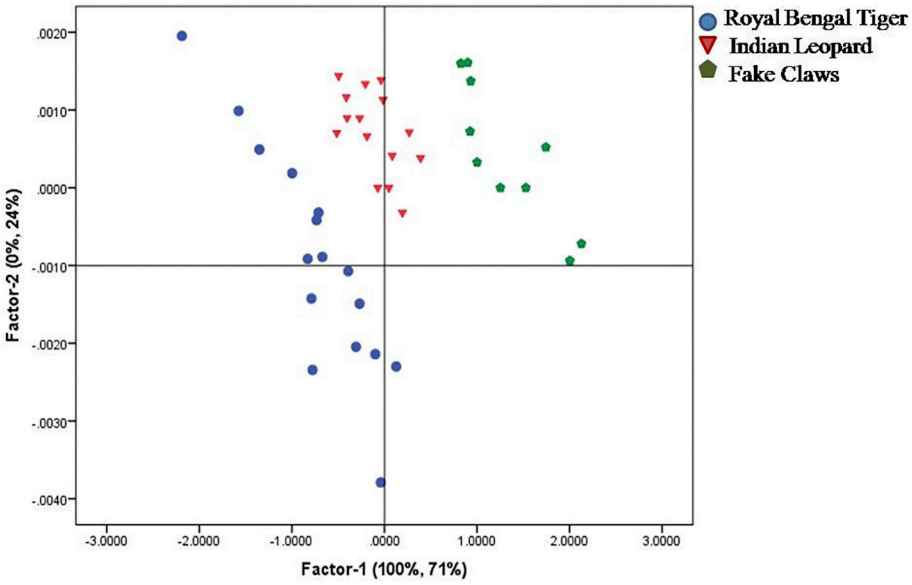

Fig. 5. PLS-DA score plot to differentiate fake claws from genuine claws of Royal Bengal Tiger and Indian Leopard class.

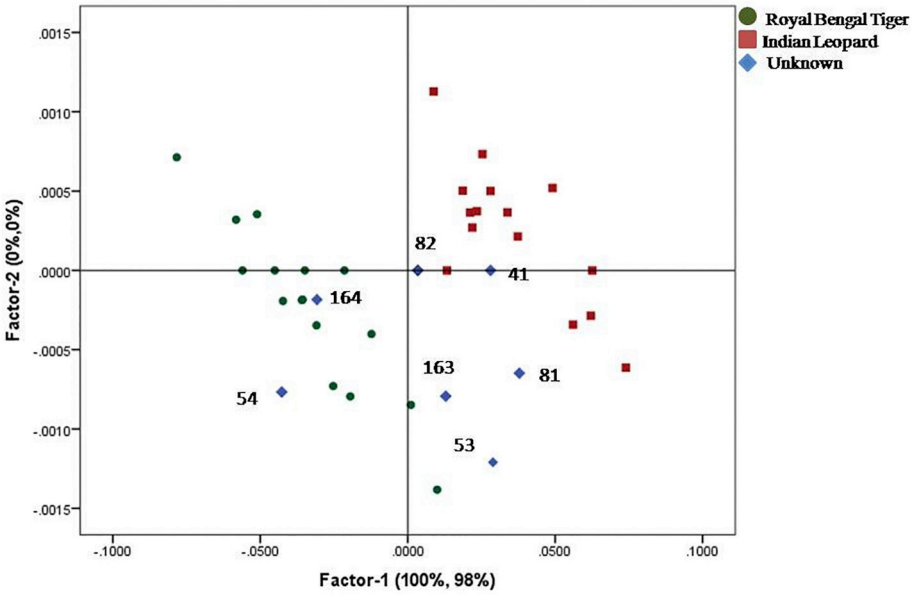

Fig. 6. Predicted Score plot to identify the blind test samples.

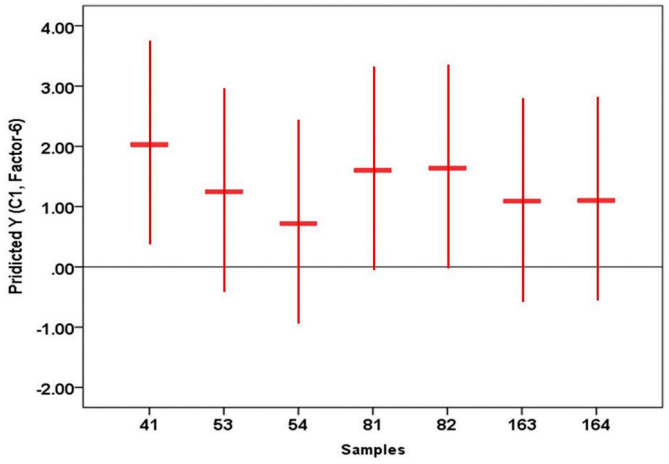

Fig. 7. Predicted Y (Claws factor) represents groups of known class (1- Royal Bengal Tiger, 2- Indian Leopard, 3 and 4- Unassigned) and X-axis represents samples of unknown claws.

Table 1
Summary of PLS-DA model predictions from blind validation test spectra.

Unknown/blind samples	Predicted class	Deviation	Actual identity
Unknown 163	1.09	0.13	1
Unknown 164	1.10	0.07	1
Unknown 41	2.03	0.07	2
Unknown 53	1.25	0.10	1
Unknown 54	0.72	0.11	1
Unknown 81	1.60	0.11	2
Unknown 82	1.64	0.09	2

Sensitivity, specificity and accuracy of this model are calculated to measure the discrimination of samples using PLS-DA.
Sensitivity = True positives/True positives + False negatives × 100.
Specificity = True positives/True negatives + False positives × 100.
Accuracy = True positive + True negative/(True positive + True negative + False positive + False negative) × 100.
The model performed 100%, sensitivity, specificity and accuracy with blind and external validation test.

Table 2
Sensitivity, specificity and accuracy values.

Claws	Sensitivity (%)	Specificity (%)	Accuracy (%)
Royal Bengal Tiger claws	100	100	100
Indian Leopard claws	100	100	100
Fake claws	100	100	100

Royal Bengal Tiger or Indian Leopard class or in general to their respective class. However for forensic scientist, there is a need to develop a method where the data from IR spectra directly be fed into an equation to predict one particular class. Such statistical models will have high forensic application in linking the claw with the endangered species as it minimizes the chances of false positive/negative results. Linear Discriminant function Analysis (LDA) is utilized for this type of classifications. The discussion and development of the model is as follow;

The absorbance values at a particular wavenumbers (cm^{-1}) that is 3266, 1634, 1540, 1509, 1469, 1452, 1418, 1393, 1338, 1232, 1112, 1071, 1052, 1024, 977, 948, 936, 893, 834, 787, 771, 746, 728, 716, 699, 665, 642, 626 and 612 are elected manually as the variables which have to be entered in the software for discriminant analysis because most of the claw samples show peaks at these wavenumbers only. The canonical discriminant function analysis has been used for classifying the claw samples into three groups that is Group I (Royal Bengal Tiger Claws), Group II (Indian Leopard Claws) and Group III (Fake Claws). The absorbance values at aforementioned wavenumber are subjected for LDA analysis. The achieved canonical correlations and eigen values are very much significant and hence, are able to classify the claw samples into their corresponding groups.

3.4.1. Analysis of variance (ANOVA)

This test allows us to select the best predictor variables for group membership. Among all the variables entered in the classification software, only the absorbance values at 1634, 1418 cm^{-1}, 1393, 1232, 1071 and 612 cm^{-1} shows significant p-values and therefore entered in the final model. It means that these absorbance values are the most appropriate for classification purposes. The developed model shows the lowest Wilks's Lambda i.e. 0.003 and 0.074 for two discriminant functions respectively with a significant p-value = 0.00. Moreover, the Box's M statistics also show significant results.

Table 3
Discriminant function's group centroids values for all three groups.

Groups	Function	
	1	2
Royal Bengal Tiger claw	−6.156	−1.830
Indian Leopard claw	0.694	4.460
Fake claw	5.461	−2.630

3.4.2. The canonical discriminant function coefficient

The discriminant function equations (DF) have to be developed by using unstandardized coefficients. A prerequisites conditions of eigen value > 1 and canonical correlation > 0.35 should be followed to develop a good model. In this study, as mentioned earlier, the model provides high eigen values that is 28.42 and 12.57 which is much greater than unity and the value of canonical correlation are 0.983 and 0.962 approaching towards the unity which are higher than 0.35 for both the discriminant functions respectively. Therefore, the developed equations will elucidate a good grouping model for unknown claw samples. The DF equations for predicting the respective claw's groups are as follow;

$$DF1 = -123.488 + (67.525 \times \text{Abs. at } 1634\ cm^{-1}) + (-55.251 \times \text{Abs. at } 1418\ cm^{-1}) + (52.885 \times \text{Abs. at } 1393\ cm^{-1}) + (-24.485 \times \text{Abs. at } 1232\ cm^{-1}) + (25.641 \times \text{Abs. at } 1071\ cm^{-1}) + (18.333 \times \text{Abs. at } 612\ cm^{-1}) \quad (1)$$

$$DF2 = -58.249 + (36.202 \times \text{Abs. at } 1634\ cm^{-1}) + (-53.220 \times \text{Abs. at } 1418\ cm^{-1}) + (5.980 \times \text{Abs. at } 1393\ cm^{-1}) + (21.70 \times \text{Abs. at } 1232\ cm^{-1}) + (15.744 \times \text{Abs. at } 1071\ cm^{-1}) + (15.321 \times \text{Abs. at } 612\ cm^{-1}) \quad (2)$$

Further, a centroid value is calculated which helps in revealing the group membership of unknown claw samples. It's basically a boundary, which uses the discriminant function scores for the classification of claw samples to their respective groups as shown in Table 3. It should be noted that the outcome of the discriminant function equation has to be examined cautiously. For example, if the values of discriminant functions come out near to −6.156 and −1.830, the sample will be grouped into Group I (Royal Bengal Tiger claw) and similarly for other two groups. The calculated value from discriminant equations for the unknown sample will closely be matched with respective groups' centroid values.

3.4.3. Classification results

It is concluded from the 'goodness of fit' model that the original classification shows 100% correct classification of all the samples in their predefined groups. Leave one out cross validation was employed to authenticate these outputs and the outcomes of cross-validation also reveals 100% accurate classification of claw samples. The calculated results represent the excellent grouping equation for unknown claws classification. All three types of samples are correctly clustered in their respective groups.

4. Conclusions

The main objective of this study was to develop a rapid and non-destructive method to differentiate Royal Bengal Tiger and Indian Leopard claws. In this study, ATR-FTIR spectroscopy combined with chemometrics is successfully used to achieve the desired objective.

The PLS-DA and LDA models both demonstrated complete separation between Royal Bengal Tiger, Indian Leopard, and fake claws whilst PCA could not discriminate between these two closely related species and fake claws as well. The external and blind validation test confirmed the classification ability of model with 100% accuracy. External validation test resulted in zero percent false positive and negative assignments for the Royal Bengal Tiger and Indian Leopard class. All samples from the blind test are assigned to their proper classes in both the models. The R-Square value obtained for each PLS-DA model for differentiating Royal Bengal Tiger, Indian Leopard and fake claws (in external validation test) is 0.99, which is highly significant for predictive accuracy.

Among both these models, LDA is having advantages over PLS-DA, because in PLS-DA, the statistical software is needed to be run every time for predicting the unknown samples to establish whether they belong to Royal Bengal Tiger or Indian Leopard class. On the other hand, the expert has two choices to select any model i.e. either PLS-DA or LDA as per the availability at his/her end for these kinds of classification problems.

In conclusion, coupling of ATR-FTIR spectroscopy with PLS-DA and LDA has a potential to present an accurate, rapid, non-destructive, reliable and an eco-friendly alternative to the existing methods for the accurate identification and differentiation among Royal Bengal Tiger and Indian Leopard claws. The present method for analysis of unknown claws could accelerate the investigation process and corroborate with the conclusions made through other examination methods.

This study offers preliminary approach of claws discrimination with limited sample size (only two species), yet provides a potential tool for such type of discrimination. This method should not be taken as a substitute to DNA based species identification, which is a well-established method for this purpose and should be treated as complementary to it.

More extensive studies covering more species with large sample size are required to explore and test full potential of ATR-FTIR spectroscopy. Nevertheless, in principle if claws of two closely related species like Royal Bengal Tiger (*Panthera tigris tigris*) and Indian Leopard (*Panthera pardus fusca*) can be differentiated then claws of distant species can also be differentiated and identified using this approach. Further, this method can also be performed on the spot (as portable ATR-FTIR instruments are commercially available), which offers quick screening of genuine/fake claw samples for the successful implementation of wildlife laws and to prevent false accusations.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Acknowledgements

The authors sincerely thank University Grants Commission (UGC), Ministry of Human Resource Development, Govt. of India for financial assistance for providing laboratory facilities in the Department of Forensic Science, Punjabi University Patiala.

References

[1] D.J. Agnew, J. Pearce, G. Pramod, T. Peatman, R. Watson, J.R. Beddington, T.J. Pitcher, Estimating the worldwide extent of illegal fishing, PLoS One 4 (2009) e4570.
[2] EUROPOL, EU Organised Crime Threat Assessment, The Hague, Netherlands (2011).
[3] United Nations Office on Drugs and Crime, The Globalization of Crime: A Transnational Organized Crime Threat Assessment, Vienna, Austria (2010).
[4] K. Krishnasamy, S. Stoner, Reduced to Skin and Bones Re-Examined: an Analysis of Royal Bengal Tiger Seizures from 13 Range Countries from 200–2015, Briefing Paper. Traffic Southeast Asia Regional Office, Petaling Jaya, Selangor, Malaysia, 2016.
[5] R.H. Raza, D.S. Chauhan, M.K.S. Pasha, S. Sinha, Illuminating the Blind Spot: A Study on Illegal Trade in Indian Leopard Parts in India (2001−2010), Traffic India/WWF India, New Delhi, India, 2012.
[6] M. Hegde, K. Suresh, K. Sivakumar, N. Krishnakumar, Indian Mammals in the Appendices of Cites a Checklist, Published by the Director Institute of Forest Genetics and Tree Breeding (Indian Council of Forestry Research and Education), 2013, www.cites.org.
[7] V. Vipin, C.P. Sharma, V.P. Sharma, S.P. Kumar, Goyal, Pioneer identification of

fake royal Bengal Tiger claws using morphometric and DNA-based analysis in wildlife forensics in India, Forensic Sci. Int. 266 (2016) 226–233.
[8] J. Manheim, K.C. Doty, G. McLaughlin, I.K. Lednev, Forensic hair differentiation using attenuated total reflection fourier transform infrared (ATR FT-IR) spectroscopy, Appl. Spectrosc. 70 (2016) 1109–1117.
[9] K. Virkler, I.K. Lednev, Blood species identification for forensic purposes using Raman spectroscopy combined with advanced statistical analysis, Anal. Chem. 81 (2009) 7773–7777.
[10] E. Mistek, I.K. Lednev, Identification of species' blood by attenuated total reflection (ATR) Fourier transform infrared (FT-IR) spectroscopy, Anal. Bioanal. Chem. 407 (2015) 7435–7442.
[11] G. McLaughlin, K.C. Doty, I.K. Lednev, Discrimination of human and animal blood traces via Raman spectroscopy, Forensic Sci. Int. 238 (2014) 91–95.
[12] G. McLaughlin, K.C. Doty, I.K. Lednev, Raman spectroscopy of blood for species identification, Anal. Chem. 86 (2014) 11628–11633.
[13] P. Bai, J. Wang, H. Yin, Y. Tian, W. Yao, J. Gao, Discrimination of human and nonhuman blood by Raman spectroscopy and partial least squares discriminant analysis, Anal. Lett. 50 (2016) 379–388.
[14] J. Fujihara, Y. Fujita, T. Yamamoto, N. Nishimoto, K. Kimura-Kataoka, S. Kurata, Y. Takinami, T. Yasuda, H. Takeshita, Blood identification and discrimination between human and nonhuman blood using portable Raman spectroscopy, Int. J. Legal Med. 131 (2017) 319–322.
[15] L. Zhang, M. Zhou, X. Li, G. Li, L. Lin, Discrimination of human and nonhuman blood using visible diffuse reflectance spectroscopy, Anal. Methods 6 (2014) 9419–9423.
[16] L. Zhang, M. Sun, Z. Wang, H. Li, Y. Li, Z. Fu, Y. Guan, G. Li, L. Lin, Optimal wavelength selection for visible diffuse reflectance spectroscopy discriminating human and nonhuman blood species, Anal. Methods 8 (2016) 381–385.
[17] L. Zhang, S. Zhang, M. Sun, Z. Wang, H. Li, Y. Li, G. Li, L. Lin, Blood species identification using near-infrared diffuse transmitted spectra and PLS-DA method, Infrared Phys. Technol. 76 (2016) 587–591.
[18] L. Zhang, M. Sun, Z. Wang, H. Li, Y. Li, G. Li, L. Lin, Noncontact blood species identification method based on spatially resolved near-infrared transmission spectroscopy, Infrared Phys. Technol. 85 (2017) 32–38.
[19] H. Lin, Y. Zhang, Q. Wang, B. Li, S. Fan, Z. Wang, Species identification of bloodstains by ATR-FTIR spectroscopy: the effects of bloodstain age and the deposition environment, Int. J. Legal Med. 132 (2018) 667–674.
[20] C.L. Pickering, J.R. Hands, L.M. Fullwood, J.A. Smith, M.J. Baker, Rapid discrimination of maggots utilising ATR-FTIR spectroscopy, Forensic Sci. Int. 249 (2015) 189–196.
[21] Q. Wang, W. Li, R. Liu, K. Zhang, H. Zhang, S. Fan, Z. Wang, Human and non-human bone identification using FTIR spectroscopy, Int. J. Legal Med. 133 (2018) 269–276.
[22] E.O. Espinoza, B.W. Baker, T.D. Moores, D. Voin, Forensic identification of elephant and giraffe hair artifacts using HATR FTIR spectroscopy and discriminant analysis, Endanger. Species Res. 9 (2008) 239–246.
[23] H. Guo, Comparative study on the infrared spectra of straight guard hairs of Indian leopard cat and Golden cat, Agric. Sci. Technol. 14 (2013) 1683–1686.
[24] G.T. Walker, Identification of animal hard tissues using Fourier transform infrared spectroscopy, Natural History Collections 17TH Triennial Conference, ICOM-CC, Melbourne, 2014.
[25] G.N. Nagaraju, Morphological studies and FT-IR analysis of skin and its appendages of wild animals, Department of Veterinary Anatomy and Histology Veterinary College, Herbal, Bangalore Karnataka veterinary, Animal and Fisheries Sciences University, Bidar, 2012.
[26] R. Kumar, V. Sharma, Chemometrics in forensic science, TrAC Trends Anal. Chem. 105 (2018) 191–201.
[27] M. Otto, Chemometrics Statistics and Computer Application in Analytical Chemistry, 3rd ed, Wiley-VCH, 2017.
[28] R. Gautam, S. Vanga, F. Ariese, S. Umapathy, Review of multidimensional data processing approaches for Raman and infrared spectroscopy, EPJ Techn. Instrum. 2 (2015).
[29] T. Naes, T. Isaksson, T. Fearn, T. Davies, Multivariate Calibration and Classification, NIR Publications, Chichester, UK, 2004.
[30] L.C. Lee, C.Y. Liong, A.A. Jemain, Partial least squares-discriminant analysis (PLS-DA) for classification of high-dimensional (HD) data: a review of contemporary practice strategies and knowledge gaps, Analyst 143 (2018) 3526–3539.
[31] L.C. Lee, A.A. Jemain, Predictive modelling of colossal ATR-FTIR spectral data using PLS-DA: empirical differences between PLS1-DA and PLS2-DA algorithms, Analyst 144 (2019) 2670–2678.
[32] D. Ballabio, V. Consonni, Classification tools in chemistry part 1: linear mofdels. PLS-DA, Anal Methods, Analyt. Methods 5 (2013) 3790–3798.
[33] N. Kanagathara, M. Thirunavukkarasu, E.C. Jeyanthi, P. Shenbagarajan, FTIR and UV-visible spectral study on normal blood samples, Int. J. Pharm. Bio Sci. 1 (2011) 74–81.
[34] C.M. Orphanou, The detection and discrimination of human body fluids using ATR FT-IR spectroscopy, Forensic Sci. Int. 252 (2015) e10–e16.
[35] J. Kong, S. Yu, Fourier transform infrared spectroscopic analysis of protein secondary structures, Acta Biochim. Biophys. Sin. 39 (2007) 549–559.
[36] J.A. Hering, P.I. Haris, FTIR spectroscopy for analysis of protein secondary structure, in: A. Barth, P.I. Haris (Eds.), Biological & Biomedical Infrared Spectroscopy: Advances in Biomedical Spectroscopy, vol. 2, 2009, pp. 129–167.
[37] S. Olsztynska-Janus, K. Szymborska-Malek, M. Gasior-Glogowska, T. Walski, M. Komorowska, W. Witkeiwicz, C. Pezowics, M. Kobielarz, S. Szotek, Spectroscopic techniques in the study of human tissues and their components. Part I: IR spectroscopy, Acta. Bioeng. Biomech. 14 (2012) 101–115.
[38] A. Barth, Infrared spectroscopy of proteins, Biochim. Biophys. Acta (BBA). Bioenerg. 1767 (2007) 1073–1101.
[39] E.O. Espinoza, B.W. Baker, C.A. Berry, The analysis of sea turtle and bovid keratin artefacts using drift spectroscopy and discriminant analysis, Archaeometry 49 (2007) 685–698.