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Review Paper

Impact of Wildlife DNA Forensics in Nepal: a short term case Review

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Abstract

Forensics in Nepal has recently started wildlife species identification using method of DNA sequencing. Although non-genetic based identification remains the first priority, sequencing technique is helpful when the sample is unidentifiable by routine methods. The article focuses on the type of wildlife evidences received and analysed by DNA sequencing in the recent years at National Forensic Science Laboratory of Nepal. The DNA based identification of wildlife materials has aided in further proceedings and prosecution.

Keywords: Wildlife crime, illegal trade, species identification, mtDNA sequencing, BLAST.

Introduction

Wildlife crime investigation is rather more complicated as compared to other forensic investigations. Wildlife forensic is essentially concerned with the identification of evidence items in order to determine the species, gender, its geographical origin or individual identity of a sample. Illegal wildlife trade is among the leading cause for the rapid decline of wildlife species. Nepal is known as the transit for illegal wildlife trade and source for some of the illegally traded species such as tiger, red panda, pangolins, leopard, deer, and elephant[1].

With the advent of DNA-based identification methods for wildlife parts and products, morphological and microscopic examinations have been limited to complete specimens only. For example, microscopy of hairs could be useful in identification of a family or genus; however, it may not yield a definitive identification[2].

For the illegally traded wildlife products which are naturally degraded or modified into other forms such as powders and oils, concise identification without molecular tools possess challenges[3]. In such cases, Antigen antibody reactions can be utilized to identify animal products such as tissue samples. However, due to lack of specific antibody to all the species and cross reactivity of non-targeted species, these tests are only considered as a presumptive in forensics[4]. The possibility of examining trace materials has been opened up by the DNA-based technologies[5]. Despite of its cost effectiveness compared to microscopy, the identification to species level can be conducted using DNA typing[6].

DNA based species identification depends on selection of markers which are preserved within the species but vary among different species[7].

The genetic loci of choice for forensic species identification are based on those derived from taxonomic and phylogenetic studies, and are primarily found on the mitochondrial genome[8]. The mtDNA are highly conserved and are present in high copy number in cell[7]. Mitochondria have a protein coat that helps protect the mtDNA from degradation. Highly degraded biological material is therefore more likely to be suitable to mtDNA typing such as typing teeth, bone or hair shafts[9-14].

The evolutionary rate of mtDNA shows that significant amount of sequence variation could be found in closely related species which is a useful feature for species identification procedures[7]. Mostly mtDNA specific region Cytochrome b (cyt b), 12S rRNA and 16S rRNA is used extensively for species identification[15-18]. The cyt b locus has been used extensively in taxonomic and forensic studies[19,20], including tiger body parts [21,22], turtle eggs and shells[23,24], crocodile skins[25], rhino horn[26], elephant ivory[27], peafowl[28] and bear bile[29,30]. There is lesser chance of misidentification using cytb compared to other locus[31]. Due to the greater sequence variation at this non-coding locus, cyt b is now being used as a tool for identifying the presence of particular species within mixture of many species [32,33].

Regardless of the locus used, the process starts with amplifying a section of the gene, the polymerase chain reaction (PCR) fragment is then sequenced directly. The species identification is achieved by comparing the sequence of target genomic region with a reference database. If there is a 100% homology, then there is confidence that the unknown sample is a member of the species to which it matches, below this confidence level, sequence variation could be due intraspecies variation, insufficient databases etc[3]. The availability of open access DNA sequence database, such as GenBank, has definitely benefited forensic science, but with certain amount of risk.

Presence of misidentified sequences[34] and lack of DNA database of endangered species requires to maintain an in house database for legal purpose.

As these sequencing approaches rely on sequencing of large DNA regions usually over 300 base pair, amplification of such large regions is difficult to achieve in degraded samples or low quality samples as in forensics, as well as some samples may produce mixed sequences as a result of contamination. Targeting of a single DNA region is also problematic as failure in the amplification of that region may give false result[3].

The isolation of genomic DNA from evidence samples becomes a crucial step for DNA analysis when samples sent in the laboratory are degraded, not well preserved or else preserved in formalin. The result may turn unreliable in case of mixed or contaminated samples as universal primer binds with all the samples[35,36]. Due to all these technical limitations when considering a forensic evidence one cannot rely upon a single method thus classical methods of identification including morphology and microscopy should be an adjunct to it[3].

Typically, items like tissues, hair, skin, scale, teeth, bone, antlers, horns, dry meat, claws, feathers, etc. are submitted to the laboratory for analysis whereas materials used to kill the animals are received rarely. In Nepal, with the technological advancement in molecular biology, wildlife forensic has gained a new insight in past few years. Now, wildlife forensic in Nepal relies on DNA sequencing for species identification rather than routine morphology and microscopy. The DNA based wildlife parts identification is the first initiative done in the country. As provision in National parks, and wildlife conservation Act, (1973) and CITES ACT (2017) of Nepal depending on nature of crime, person convicted in wildlife crime is liable to 5-15 years of imprisonment or a fine of NRs 50,000 to one million rupees or both[37,38]. However, mtDNA has maternal origin of inheritance and if a hybrid species is protected by law and it is produced by using non- protected maternal animal species, the mtDNA analysis will result in the profile of maternal non-protected species[7] which is problematic for identification as well as it may create a barrier for the current rule of imprisonment and fine. The aim of this article is to highlight technical significance of wildlife DNA forensics and its impacts on wildlife crime investigation for concerned law enforcement agencies based on a total of 80 wildlife exhibit analyzed in the laboratory.

The procedure from isolation of DNA to identification is summarized below in the flowchart. Different suspected wildlife sample received and analyzed in the laboratory by DNA sequencing is shown in the Table-1.

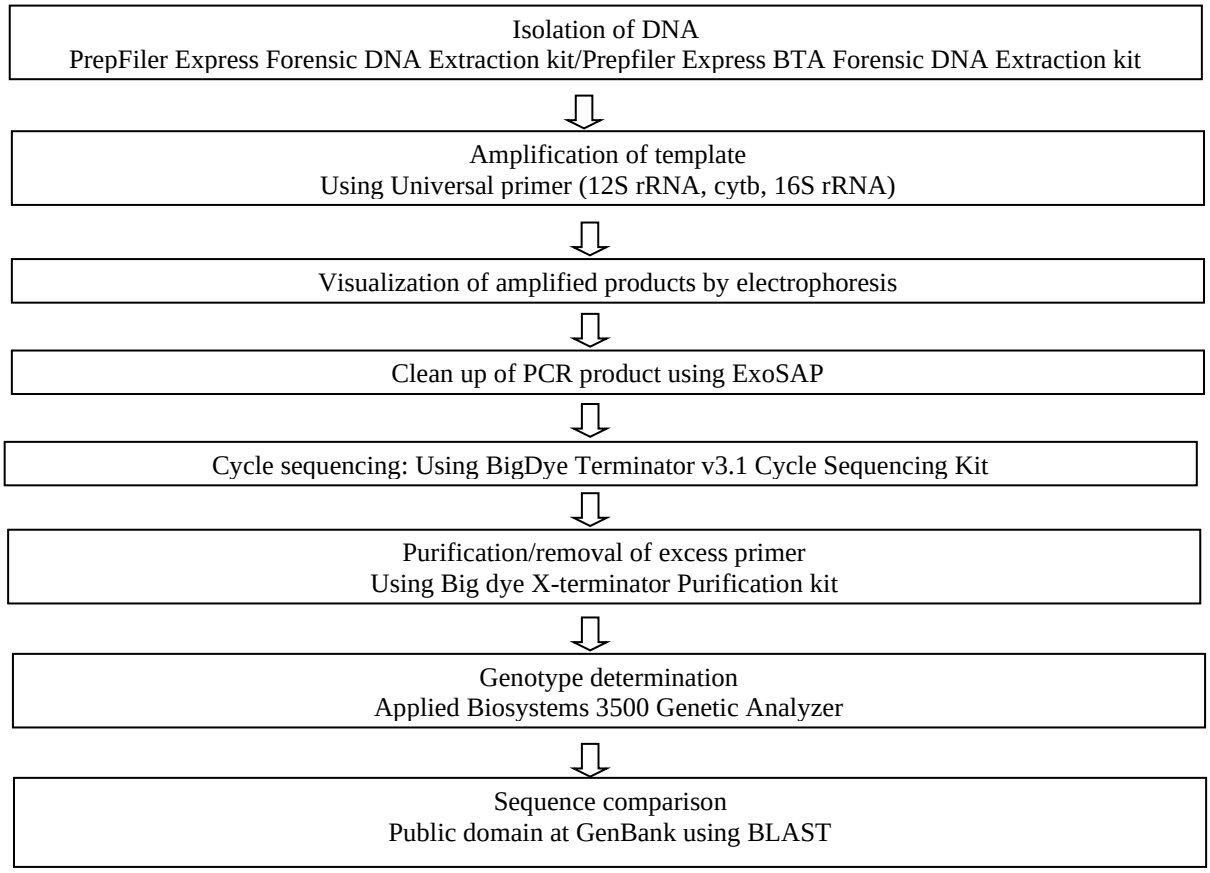


Figure-1: Procedure for isolation and sequencing.

Table-1: Suspected Cases of soft tissues.

Suspected items (N)	Species Identified : Common/ Scientific name (N)	Gene bank Accession number	Homology (%)	CITES appendix
Bear gall bladder (18)	Asian black bear/ *Ursus thibetanus* (9)	MG066704.1,FM177759.1,MG066704.2	100	I
	Buffalo/*Bubalus bubalis* (7)	KX758295.1	100	-
	Goat/*Capra hircus* (1)	KX758295.1	100	-
	Goral/ *Naemorhedus goral* (1)	FM179469.1	100	I
Meat/ tissues (21)	Asian house shrew/ *Suncus murinus* (1)	AF434824.1	98.44	-
	Barking deer/ *Muntiacus muntjak* (4)	KU709862.1,AM778453.1,KY117560.1	100	-
	Chital/ *Axis axis* (4)	JN093075.1, KP755027.1	100	-
	Goat/ *Capra hircus* (2)	MH229952.1	100	-
	Goral/ *Naemorhedus goral* (1)	FM179469.1	100	I
	Himalayan tahr/ *Hemitragus jemlahicus* (2)	KF317937.1	99	-
	Indian crested porcupine/ *Hystrix indica* (1)	KX611874.1	99.39	-
	Langur/ *Semnopithecus entellus* (1)	EU004478.1	99	I
	Leopard Cat/ *Prionailurus bengalensis* (1)	KX857795.1	100	II
	White stork/*Ciconia ciconia* (1)	DQ485798.1	98.58	-
	Wild Boar/ *Sus scrofa* (2)	JN861025.1, NC039090.	99	-
	Yak/ *Bos mutus and Bos grunniens* (1)	KY829451.1, KX232527.1	100	?
Degraded/ piece of skin (7)	Barking Deer/*Muntiacus muntjak* (1)	AM778453.1	100	-
	Himalayan blue sheep/*Pseudois nayaur* (1)	JX101652.1	100	-
	Muskrat/*Ondatra zibethicus* (1)	KU177045.1	100	-
	Leopard cat/*Prionailurus bengalensis* (1)	KX857795.1	100	II
	Small indian civet/ *Viverricula indica* (1)	KX891749.1	96.86	-
	Tiger/ *Panthera tigris* (1)	JX101607.1	100	I
	Yellow bellied Weasel/*Mustela kathiah* (1)	HM106320.1	99	-
Hair (2)	Common Leopard /*Panthera pardus* (1)	JX101609.1	100	I
	Tiger/*Panthera tigris* (1)	MH538949.1	100	I
Musk Pod skin (2)	Goat/*Capra hircus* (1)	MH165339.1	99	-
	Musk Deer/ *Moschus leucogaster, Moschus chrysogaster, Moschus fuscus* (1)	NC042604.1,KP684123.1,EF219402.1	100	I
Snout (1)	Wildboar/*Sus scrofa cristatus* (1)	MG725631.1	100	-

Table-2: Suspected cases of Exoskeleton and Endoskeleton.

Suspected items (N)	Species Identified : Common / Scientific name (N)	Gene bank Accession number	Homology (%)	CITES appendix
Antler/ Horn (5)	Buffalo / *Bubalus bubalis* (1)	KX758295.1	100	-
	Chital / *Axis axis* (1)	JN093075.1	99	-
	Hog Deer / *Axis porcinus* (1)	KP755033.1	100	-
	Sambar Deer / *Cervus unicolor* (1)	AY184434.1	100	-
	Yak / *Bos grunniens* and *Bos mutus* (1)	MH921427.1, CP027082.1	100	?
Paws (2)	Clouded leopard / *Neofelis nebulosa* (1)	KP201191.1	100	I
	Himalayan black bear / *Ursus thibetanus* (1)	FM177759.1	99.45	I
Claws (5)	Common Leopard / *Panthera pardus* (1)	MH588632.1, KP001507.1	92	I
	Crested serpent eagle / *Spilornis cheela* (1)	JN191388.1	100	-
	Himalayan black bear / *Ursus thibetanus* (1)	FM177759.1	100	I
	Jungle cat / *Felis chaus* (1)	KU963205.1	98	
Canine (4)	Clouded leopard /*Neofelis nebulosa* (2)	KP202291.1	100	I
	Himalayan black bear / *Ursus thibetanus* (2)	EF667005.1, FM177759.1	100	I
Elephant tusk (1)	Sambar deer / *Rusa unicolor* (1)	MF177027.1	92.33	-
Skin exuviate (1)	King Cobra /*Ophiophagus hannah* (1)	KX694860.1	99	II
Feathers (1)	Crimson horned pheasant / *Tragopan satyra* (1)	AF200724.1	99	III
Skull (4)	Chinese pangolin / *Manis pentadactyla* (1)	AY012154.1	97.59	I
	Chital / *Axis axis* (1)	KP755027.1	100	-
	Common Leopard / *Panthera pardus* (1)	MK842148.1	100	I
	Gray langur / *Seminopithecus entellus*	AF420043.1	97	I
Femur (1)	Common Leopard / *Panthera pardus* (1)	JX101609.1	100	I
Scapula (1)	Common Leopard / *Panthera pardus* (1)	MK842148.1	100	I
Flat Bone (1)	Crested serpent eagle / *Spilornis cheela* (1)	JN191388.1	97	-
Leg part (2)	Goral / *Naemorhedus goral* (1)	FM179469.1	100	I
	Peacock / *Pavo cristatus* (1)	AY722396.1	99.75	-
Spine (1)	Porcupine / *Hystrix indica* (1)	KX611874.1	99.73	-

Results and Discussion

Wildlife crime investigation in recent years relies on DNA testing for species identification in Nepal. Before the new technological developments morphology and microscopy based approaches were used which are still considered in the analysis.

The suspected cases of Bear Gall bladder (Table-1) was sequenced using 12S rRNA gene of mitochondrial DNA, 9 suspected samples showed 100% similarity with Asian black bear, 7 with Buffalo and remaining 2 with Goral and Goat. Bear bile which has been an important constituent in Traditional Asian Medicine is obtained by hunting Asian black bear for their Gall bladders[29,39]. International trade of *Ursus thibetanus* is prohibited, it is classified as vulnerable by the world conservation union and listed on appendix I CITES[40,41]. Several techniques have been used for species identification of suspected bear Gall bladder which can distinguish genuine bear derivative from those of pig or goat but cannot distinguish *Ursus thibetanus* parts with other bear species[42,43] which is important in legal framework. Genetic techniques provide powerful tools for wildlife forensic and can provide definitive species identifications; however the analysis is often complicated by the existence of fraud items, and the suspected item is also identified as domestic pig, buffalo or goat by laboratory analysis[44,45].

Wild meat has been an important source of protein for humans in ancient times[46] in south Asian countries including Nepal. Traditional hunting of wildlife to meet the cultural needs has been practiced[47,48]. Despite of laws forbidding hunting, wildlife meat continues to fulfill the needs of many local communities [48]. Among different muscle tissue samples obtained in the laboratory and subjected for sequencing the most common is of Deer family such as barking deer and chittal.

Suspected muscle sample (Table-1) showed 99% similarity with Wild boar (*Sus scrofa)* when 415 base pair sequence of mitochondrial DNA Cytb gene was amplified, but could not be identified at subspecies level. In a study from eastern India DNA data using cytb gene identified the samples to be of *Sus scrofa* but subspecies could not be confirmed[51]. Indian wild boar (*Sus scrofa cristatus)* is a subspecies of wild boar native to India, Nepal, Burma, Western Thailand and SriLanka, it is a protected species and is often hunted illegally. Indian wild pig has been differentiated from other wild pig races using the sequence generated from partial fragment (421 base pair) of mitochondrial DNA cytb gene[52].

DNA techniques have been proved to be the benchmark in species identification, however due to lack of reference DNA sequences from ancient wild and domesticated species causes the sequencing reports to be incomplete sometimes, as observed in case of Yak (Table-1). The domestic Yak from Nepal, Tibet and China was first referred to as *Bos grunniens*[53], the wild Yak *Bos mutus* has been protected under CITES[54]. Wild Yak can be distinguished from its domestic counterpart on the basis of its body sizes, with domestic Yak always being much smaller than wild Yak[53]. Molecular and cytogenetics of both domestic and wild Yak has been intensively reviewed, but to understand the evolutionary relationship between wild and domestic Yak a good number of sample is required[55,56].

Himalayan tahr (*Hemitragus jemlahicus*) which is native to Himalayas in southern Tibet, northern Pakistan, northern India and Nepal is listed as near threatened on the IUCN red list as the population is declining due to habitat loss and hunting[57]. The species has been identified in the muscle tissue sample from testis amplifying 381 basepair sequence of mitochondrial DNA12S rRNA gene (Table-1). The generated 12S rRNA sequence of suspected antler samples showed similarities with database sequence of Buffalo, Yak and Deer species (Table-2).

(a) Suspected horn (b) Suspected hoof

Figure-2: Suspected Rhinoceros parts.

High demand for Rhino horn products in Asia is the reason for increasing Rhino Poaching[58]. A forensic test is conducted to determine whether the suspected horn is actually from Rhino or from different species. Suspected Rhinoceros horn and hoof (Figure-2) received in the laboratory were subjected for DNA isolation but no amplicons were produced. In a study by Kyle M et al[58], ninety percent of seized horn specimens were identified using universal Rhino primers where five horns were reported as no result due to fake or substitute horns. Sometimes fake horns are manufactured as non-biological materials which do not work with universal primers.

Microscopy of animal hair alone cannot be used to identify the species[59]. In such cases the DNA based identification might be valuable. The generated sequence data of 12S rRNA of two separate hit and run case samples and its comparison with reference data available in Genbank showed 100% similarity with *Panthera tigris* and *Panthera pardus* (Table-1). Tiger and their close relatives (Panthera) are some of the world's most endangered species[60]. Bengal tigers in Nepal are found in fragmented habitats in the southern plain of the country and has been declining mainly due to poaching[61]. The sequence generated from 12S rRNA of suspected piece of skin sample (Table-1) also showed 100% similarity with *Panthera tigris*. Suspected skin sample (Table-2) which was obtained in the form of skin exuviate (Figure-3) showed 99% similarity with *Ophiophagus hannah* (King Cobra) using cytb primer. Skin exuviate can be a good noninvasive source of DNA and can be used for DNA barcoding[62]. King Cobra is world largest venomous snake and is listed as Vulnerable in IUCN red list[63].

Snakes possess adaptive characters in various aspects of morphology, which may cause misinterpretation in identification[64].

Figure-3: Suspected skin exuviate.

Identification becomes challenging when the sample seized is in modified form. The seized material received in the laboratory was a wearing cap (Figure-4) and source of DNA was skin. The generated 12S rRNA sequence showed 100% similarity with Muskrat (*Ondatra zibethicus*) (Table-1).

Figure-4: Suspected Cap.

Muskrat is a semi aquatic rodent found in North America, Europe, and Asia. It is considered as a precious domesticated animal based on its fur of economic importance and perfume secreted by male muskrat[65,66].

Different claws sample received as a part of forensic wildlife case investigation (Table-2) were subjected for DNA analysis using 12S rRNA, the generated sequence showed 100% similarity with database sequence of Himalayan black Bear (*Ursus thibetanus*), Common leopard (*Panthera pardus*), and Jungle cat (*Felis chaus*). However, the generated sequence of 12S rRNA derived from Keratin material of claw showed only 92% similarity with Crested serpent Eagle (*Spilornis cheela)* (Table-2) probably due to degraded sample and comparatively short base pair sequence generated. Some of the differences observed between unknown and reference samples may be the result of subsequent modification in DNA sequence due to cell death[67,68]. The generated 12S rRNA sequences of four suspected canine sample (Table-2) showed similarities with Asian black Bear (*Ursus thibetanus*) and Clouded Leopard (*Neofelis nebulosa*). DNA isolated from suspected Elephant tusk (Table-2) and sequenced using 12S rRNA gene showed 92.33% similarity with Sambar Deer (*Rusa unicolor*).

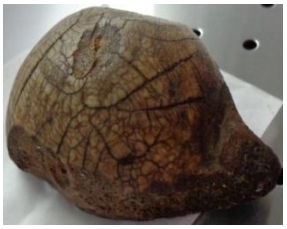
Genuine ivory being an expensive luxury item is difficult to obtain. To protect threatened species like Elephants commercial trading of ivories is banned which is the main reason for ivories imitations and fakes to increase. Ivory alike products are sold at low prices. Multiple tests are used for the identification of ivories which includes use of black light, presence of unique grain structure, schreger lines[69]. DNA based methods are currently used for the identification of ivories. It is reported that ivory samples have lower success rate (about 55%) of obtaining a PCR product compared to tissue. It is expected that the DNA within ivory will be severely degraded limiting the amount and quality of DNA that can be extracted[70]. Figure-5 shows the suspected elephant tusk received in the laboratory from which isolation of DNA was not possible.

Figure-5: Suspected Elephant Tusk.

Hard tissue is one of the most difficult biological samples used for DNA extraction. Different such material received in the laboratory includes skull, femur, scapula, flat bone, leg and spine. Generated 12S rRNA and 16S rRNA sequence showed similarities with reference database sequence of Chinese pangolin (*Manis pentadactyla*), Common Leopard (*Panthera pardus*), Crested serpent Eagle (*Spilornis cheela*), Chittal (*Axis axis*), Goral (*Naemorhedus goral*), Gray Langur (*Seminopithecus entellus*) Peacock (*Pavo cristatus*), Porcupine (*Hystrix indica*).

a)

b)



c)

Figure-6: Suspected tooth, bone and carapace materials.

Figure-6 Shows the suspected materials received in the laboratory where a) was suspected Tooth b) was the piece of long bone, b) and c) was suspected to be turtle shell. Meaningful DNA sequencing was not possible from these samples. Analysable DNA often persists in bones and teeth much longer than in the soft tissues of the body. DNA molecule remains bonded with hydroxyapatite of hard tissues which stabilizes it for longer period of time; however the process of reprecipitation and dissolution releases DNA vulnerable for degradation.

In addition solutes incorporated into the bone and teeth from soil minerals serve as PCR inhibitor that limit subsequent DNA analysis[71,72].

Many birds and bird products such as Feathers are protected under the US Migratory Bird treaty (MBTA), the US Endangered Species act (ESA) and the CITES[72]. Mitochondrial DNA based identification of feather barbs was possible for as few as two feathers[73]. The generated mitochondrial DNA 12srRNA sequence of seized feather sample showed 99% similarity with Crimson horned pheasant (*Tragopan satyra*), Table-1, Figure-7a.

a) b)

Figure-7: Feathers.

It is a Pheasant found in Himalayas reaches of Nepal, India, Tibet, and Bhutan and is listed as near threatened in IUCN redlist[74]. Feather can be a reliable DNA substrate, unless the feather is damaged, dyed, or present in the laboratory partially. Down Feathers were received in the laboratory as seized feather sample from which DNA isolation was not possible (Figure-7b).

Musk Deer are threatened primarily due to illegal trade of muskpod which is used to produce perfumes. A 250bp sequence of 12S rRNA generated by isolation of DNA from skin portion of suspected muskpod (Table-1) showed 100% similarity with reference database sequence of Musk Deer. Recent studies showed seven species of Musk Deer are known to occur in the forests and alpine scrublands of the mountains of Asia. Three species Alpine musk Deer (*Moschus chrysogaster*), Himalayan musk Deer (*Moschus leucogaster*), and Black musk deer (*Moschus fucus*) have so far been reported from Nepal[75]. Similarly DNA sequence generated from hair root portion of suspected muskpod showed 99% similarity with *capra hircus*.

Conclusion

DNA assists in investigations where identification of samples is not possible from routine methods. Since the introduction of DNA facility, a total of 29% cases (N= 68) have been examined through DNA analysis. Though this ratio is low, identification of 8 CITES I listed species (Table-1 and 2) definitely shows the impact of wildlife DNA forensics to law enforcement authorities. However, reliable identification of hybrids is still problematic. The problem also arises in case of highly degraded sample which produces shorter amplicons such that the chance of closely related species sharing the same sequence or having high homology scores increases. Therefore, it can be concluded that in this new era, DNA-based identification methods will be a boon for developing countries like Nepal in wildlife species identification for forensic laboratories and ultimately in legal procedures.

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