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Utility and Applicability of a Universal Set of Primers in Identifying the Sex of South and Southeast Asian Mammals

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Sex identification of individuals is an important task in wildlife forensics as well as in conservation biology. It helps scientists understand population sex ratios with respect to maintaining genetic diversity, managing inbreeding depression and preventing the demographic consequences of sex-biased poaching. The literature on the use of mammalian molecular sex markers indicates that the success of accurate sex identification is variable across species. Very little is known about the effectiveness of such markers on the mammals of South and Southeast Asia. Therefore, we selected and tested three sets of universal primers for low-cost gel-based sex identification of mammals. We amplified different sets of markers—SRY (157 bp) and 12S rRNA (384 bp); Y-53-SRY (225 bp) and ZFX/ZFY (P1/P2; 445); SRY (157 bp) and 12S rRNA (151 bp)—to be used with different types (tissue, hair and skin) of samples from 20 mammalian species. All three sets of primers amplified the sex-specific fragment in a range of samples from hair to tissue. With an increasing number of field studies using non-invasively collected samples, this proposed low-cost gel-based method of molecular sexing may be applied in various aspects of the ecology and biology of South and Southeast Asian mammals, their conservation and forensics. We suggest that at least two sets of primers be used for any biological samples to avoid ambiguity.

Key words: Sex identification, Duplex PCR, Internal marker, Wildlife forensic science, Mitochondrial DNA.

BACKGROUND

Identification of the sex of mammals from a population facilitates behavioural, breeding system and evolutionary ecology studies of different species (Rosel 2003). Sex identification is also crucial for understanding the effects of sex-selective harvesting in sports and in dealing with the illegal trade of wildlife species of high conservation priority (Spong et al. 2000; Milner et al. 2007; Mondol et al. 2014), as a skewed population sex ratio may affect the mating system and biology of a species (Mysterud et al. 2002). Samples collected from the field provide information regarding the population dynamics and demographic structure of a species as well as sex-biased habitat use by the species (Brown et al. 1991; Gompper et al.1998; Hughes 1998; Eggert et al. 2003). Therefore, many markers and techniques have been proposed to identify the sex of an individual. These include cytogenetic analysis, detection of H-Y antigen and measurement of X-linked enzymes before Barr body formation (Bondioli 1992). PCR-based sex identification can be performed using random fragment length polymorphisms (RFLP) (Aasen and Medrano 1990; Palsboll et al. 1992), TaqMan Probe-based real-time PCR (Chou et al. 2010), fluorescent labelled sex-specific primers (Settin et al. 2008; Mukesh et al. 2013), and hybridization and ligation (Zoledziewska and Dobosz 2003). The random amplified polymorphic DNA (RAPD) technique has also been used for gender determination, but this technique has not been found

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to be reliable for sexing mammalian species because it is time consuming and there is a chance that the sex-specific band disappears during the reaction (Smith et al. 1994).

PCR has been used for sex determination by targeting sex-specific regions in the genome such as the sex-determining Y chromosome (SRY) gene (Pomp et al. 1995; McHale et al. 2008; Han et al. 2010), amelogenin (Sullivan et al. 1993) and the zinc finger protein encoding Zfx and Zfy genes (Aasen and Medrano 1990). Among these, SRY has been used for many mammalian species (Pomp et al. 1995; Garcia-Meunier et al. 2001; Mukesh et al. 2013). Successful amplification of the Y-specific region indicates male identity. However, in poor-quality samples (hair, degraded tissues, museum specimens and fecal samples) and even sometimes in samples of good quality, PCR may fail to amplify DNA due to the presence of an inhibitor or the amount of DNA and not due to the animal being female (Ortega et al. 2004). Some proposed solutions to this include co-amplification of mitochondrial DNA or a single copy of a nuclear gene in duplex PCR along with a sex-specific fragment in a single reaction (Kamimura et al. 1997; Ortega et al. 2004). A homologous gene, such as amelogenin, present in both X and Y chromosomes in mammalian species producing different-sized amplicons in males and females have also been used in sex identification. But the usability of this gene for sexing in some species of mustelid has not been confirmed (Hattori et al. 2003). However, Y-specific genes have also failed to amplify in some rodent species (Bryja and Konečný 2003). On the other hand, zinc finger proteins can be amplified using a single set of primers that are specific for identifying the sex of mammalian species (Palsboll et al. 1992; Aasen and Medrano 1990; Ortega et al. 2004; Xu et al. 2009). Most of the sex identification methods have been explored in the felids and other carnivores and only a few have been tested on other mammals (Wei et al. 2008; Mukesh et al. 2013; DeCandia et al. 2016).

In view of the mixed findings regarding molecular sexing in the literature, we explored the use of PCR-based sex identification of mammals using the Y-specific gene. Most of these species are threatened by several factors such as illegal hunting, habitat destruction and climate change (Woodroffe 2000; Check 2006; Corlett 2007; Shepherd 2008). These threats affect their size and alter their population structures (Ginsberg and Milner-Gulland 1994). Our main focus in this study was to test and optimize the use of published universal primer sets for sex identification and suggest the applicability of these primer sets in identifying the sex of mammals distributed in South and Southeast Asian countries. Many biological samples obtained during field surveys, including surveys of elusive species, as well as wildlife forensics samples, are of poor quality and yield low-quality DNA (Taberlet et al. 1999; Teletchea et al. 2005; Pages et al. 2009), making the amplification of large fragments unreliable. Therefore, targeting smaller amplicon sizes in the sample types mostly encountered in the wildlife forensics—*e.g.* non-invasively collected or preserved in the formalin (Teletchea et al. 2005; Brinkman and Hundertmark 2009; Joshi et al. 2013)—increases the chance of amplification success from templates with low copy numbers. Therefore, we tested the applicability of a gradient of small to large (151–445 bp) amplicons using both degraded and good-quality samples from different mammals found in South and Southeast Asia.

MATERIALS AND METHODS

Selection of sex primers

We identified some robust primers based on their success in sexing and species identification as described in the literature. We selected universal primer sets of three markers 151–445 bp long (Table 1). Two mitochondrial primers (12S rRNAs) of different sizes and one ZFX-ZFY homologous sex marker (P1/P2) (Aasen and Medrano 1990) were used as internal markers to avoid the non-amplification of sex primers due to the presence of PCR inhibitors or low quality DNA, as recommended in the literature (Ortega et al. 2004).

The primers were selected based on the fact that most samples recovered from the wildlife trade (such as processed meat, bones, claws, tanned skins, carrion, hair, horn and ivory) are of poor quality (Jackson 1990; Mills 1993; Wasser et al. 2008; Milliken and Shaw 2012; TRAFFIC 2011). Such samples are difficult to amplify due to their prolonged exposure to ambient conditions, resulting in degradation of DNA through autolysis and bacterial action (Lindahl and Andersson 1972). Thus, only gene fragments of < 500 bp from these samples should be used to identify sex and species (Goyal et al. unpublished data). Therefore, we selected small primers with one internal marker (< 450 bp), which is within the range of most forensic and biological samples collected from field surveys or museum samples. Sex was identified based on the amplification of Y-linked sex-specific bands co-amplified with internal primers (12S rRNA and P1/P2), as suggested in the literature, to avoid 'false positives' for female samples (Ortega et al. 2004). This technique was used for both male and female animals, as two bands appeared in samples from male animals, one band being that of a Y-linked

sex-specific primer and the second one being that of an internal primer. In samples from female animals, only the internal primer was amplified.

DNA extraction and quality assessment

We selected samples from 20 Indian mammal species that are also distributed in other parts of South and Southeast Asia and often reported to be affected by trade and habitat fragmentation. Species that we tested for sex identification are distributed in South and Southeast Asia and selected samples included tissues, hair and tanned skins (Table 2). The study was conducted from 2011–2013.

DNA was extracted from the tissue samples using the Qiagen Tissue Kit according to the manufacturer's protocol. We made some modifications to the protocol to extract DNA from the tanned skins. In the cases of tanned skins, Proteinase K (20 µl/ml) was added at 8–12 hour intervals and the samples were incubated for a long period (2 days) to increase the yield of DNA in the final extract. After the skins were digested completely, DNA was extracted according to the manufacturer's protocol. DNA was extracted from the hair samples by chopping the hair into small pieces and placing the pieces into sterilized 1.5 mL Eppendorf tubes. Lysis buffer (Tris-Cl (10 mM), EDTA (10 mM), NaCl (100 mM)), 2% sodium dodecyl sulphate (SDS), 10 µl of Proteinase K and 10 µl of dithiothreitol (DTT, 10 mM) were added. The samples were incubated at 56°C in a water bath for 8 hours. Additional Proteinase K and DTT (10 µl each) were added during the incubation phase to digest the hair faster. After 8 hours, the samples were removed from the water bath and centrifuged at 8000*g* for 1 minute. The subsequent steps were carried out using the Qiagen Tissue Kit according to the manufacturer's protocol. The quality of the DNA was tested on 0.8% gel and quantified using a 1 kb ladder (HiMedia). All the DNA samples were verified using gel electrophoresis and categorized as 'good', 'moderate', or 'low' quality or 'no visible DNA' (Fig. 1). Good-quality DNA was diluted 1:100 with elution buffer to reduce the concentration. Moderate-quality DNA was diluted 1:50, and low-quality DNA was diluted 1:20. Samples with no visible DNA were used directly (1 µl) to obtain a clear band. These were undertaken because failure to amplify the targeted genes may also be due to a high concentration of DNA or the presence of an inhibitor (Bryja and Koncny 2003).

PCR amplification of sex primers

Sex identification of 20 mammalian species was performed using three sets of sex primers with one internal primer (Table 2). One known male positive sample and one female positive sample were used as controls during the PCR amplification of these sets of primer. The amplification of sex primers was performed with 2.5 mM $MgCl_2$, 0.2 µM SRY primers, 0.3 µM12S

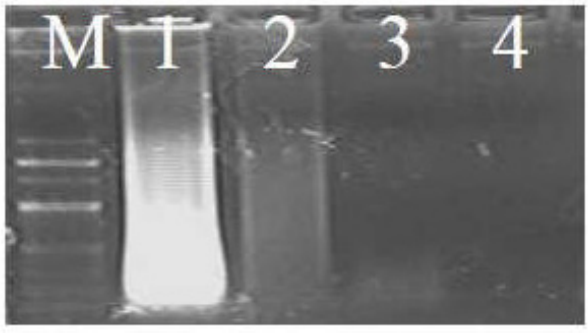

Fig. 1. Quality of DNA extracted on 0.8% agarose gel. M, 100 bp leader; 1, good quality; 2, moderate quality; 3, low quality; 4, no visible DNA.

Table 1. List of primer sets used in this study

Set	Primer	Size (bp)	Primer	Primer sequence	Reference
I	12S rRNA	384	L1091	5′-AAA AAG CTT CAA ACT GGG ATT AGA TAC CCC ACT AT-3	Kocher et al. 1989
			H1478	5′-TGA CTG CAG AGG GTG ACG GGC GGT GTG T-3′	
	SRY	157	SRYA-5	5′-TGAACGCAGTCATGGTGTGGT-3′	Pomp et al. 1995
			SRYA-3	5′-AATCTCTGTGCCTCCTGGAA-3′	
II	ZFX/ZFY	445	P15EZ	5′-ATAATCACATGGAGAGCCACAAGCT-3′	Aasen and Medrano 1990
			P13EZ	5′-GCACTTCTTTGGTATCTGAGAAAGT-3′	
	SRY	225	Y53-3C	5′-CCCATGAACGCATTCATTGTGTGG-3′	Fain and LeMay 1995
			Y-53-3D	5′-ATTTTAGCCTTCCGACGAGGTCGATA-3′	
III	12S rRNA	151	12Sa	5′-CTG GGG ATT AGA TAC CCC ACTA-3′	Rohland et al. 2004
			12So	5′-GTC GAT TAT AGG ACA GGT TCC TCT A-3	
	SRY	225	Y53-3C	5′-CCCATGAACGCATTCATTGTGTGG-3′	Fain and LeMay 1995
			Y-53-3D	5′-ATTTTAGCCTTCCGACGAGGTCGATA-3′	

Table 2. PCR amplification with different sets of primers (+) and reported distribution of mammalian species in South and Southeast Asian countries

SN	Species	Common name	N	Set I	Set II	Set III	Southeast Asian countries										
							BR	CA	ET	IN	LA	MA	MY	PH	SI	TH	VI
Wild species[1]																	
1	*Panthera tigris*	Tiger	3[#]	+	+	+(2)				*			*			*	
2	*Panthera pardus*	Leopard	7[†]	+	+(51)	+(2)		*		*		*	*		*	*	*
3	*Prionailurus bengalensis*	Leopard cat	2[‡]	+	+	+	*	*		*		*	*	*	*	*	*
4	*Lutra* sp.	Otter	4[†]	+	+ (89)	+(3)		*		*			*			*	*
5	*Antilope cervicapra*	Blackbuck	2[‡]	+	+	+											
6	*Boselaphus tragocamelus*	Nilgai	2[‡]	+	+	+(4)											
7	*Bos gaurus*	Gaur	2[‡]	+	+	+		*				*	*			*	*
8	*Muntiacus muntjak*	Barking deer	2[‡]	+	+	+	*			*		*			*	*	
9	*Capricornis thar*	Serow	2[†]	+	+	+											
10	*Naemorhedus goral*	Goral	2[‡]	+ (14)	+ (31)	+											
11	*Hemitragus jemlahicus*	Himalayan tahr	2[‡]	+	+	+											
12	*Gazella bennettii*	Chinkara	2[‡]	+	+	+											
13	*Axis porcinus*	Hog deer	2[‡]	+	+	+		*		*							
14	*Rusa unicolor*	Sambar	2[‡]	+	+	+(20)	*			*		*	*			*	*
15	*Elephas maximus indicus*	Elephant	3[#]	+	+	+(2)		*		*		*	*			*	*
16	*Sus scrofa*	Wild pig	2[‡]	+	+	+		*		*		*				*	*
Domestic species																	
17	*Bubalus bubalis*	Buffalo	2[‡]	+	+	+											
18	*Equus ferus caballus*	Horse	2[‡]	+	+	+											
19	*Bos taurus*	Cow	2[‡]	+	+	+											
20	*Capra aegagrus hircus*	Goat	2[‡]	+	+	+											
Total number of wild species found in countries			44	14	171	33	3	7		9		7	7	1	3	9	7

SN	Species	Common name	N	Set I	Set II	Set III	South Asian countries							
							AF	BA	BH	IND	MAL	NE	PA	SR
Wild species[1]														
1	*Panthera tigris*	Tiger	3[#]	+	+	+(2)		*	*	*		*		
2	*Panthera pardus*	Leopard	7[†]	+	+(51)	+(2)	*	*	*	*		*	*	*
3	*Prionailurus bengalensis*	Leopard cat	2[‡]	+	+	+	*	*	*	*		*	*	
4	*Lutra* sp.	Otter	4[†]	+	+ (89)	+(3)	*	*	*	*		*	*	*
5	*Antilope cervicapra*	Blackbuck	2[‡]	+	+	+		*		*		*	*	
6	*Boselaphus tragocamelus*	Nilgai	2[‡]	+	+	+(4)		*		*		*	*	
7	*Bos gaurus*	Gaur	2[‡]	+	+	+		*	*	*				*
8	*Muntiacus muntjak*	Barking deer	2[‡]	+	+	+				*				
9	*Capricornis thar*	Serow	2[†]	+	+	+		*	*	*		*		
10	*Naemorhedus goral*	Goral	2[‡]	+ (14)	+ (31)	+			*	*		*	*	
11	*Hemitragus jemlahicus*	Himalayan tahr	2[‡]	+	+	+				*		*		
12	*Gazella bennettii*	Chinkara	2[‡]	+	+	+				*			*	
13	*Axis porcinus*	Hog deer	2[‡]	+	+	+		*		*		*	*	
14	*Rusa unicolor*	Sambar	2[‡]	+	+	+(20)		*	*	*		*		*
15	*Elephas maximus indicus*	Elephant	3[#]	+	+	+(2)	*	*	*	*		*	*	*
16	*Sus scrofa*	Wild pig	2[‡]	+	+	+	*	*	*	*		*		*
Domestic species														
17	*Bubalus bubalis*	Buffalo	2[‡]	+	+	+								
18	*Equus ferus caballus*	Horse	2[‡]	+	+	+								
19	*Bos taurus*	Cow	2[‡]	+	+	+								
20	*Capra aegagrus hircus*	Goat	2[‡]	+	+	+								
Total number of wild species found in countries			44	14	171	33	5	12	10	16		13	9	6

[1]Based on native range description in IUCN Red List. N, Number of samples. [‡]Tissues. [†]Tanned skins. [#]Hair and tissue. Values in parentheses indicate additional samples tested against respective species. BR, Brunei; CA, Cambodia; ET, East Timor; IN, Indonesia; LA, Laos; MA, Malaysia; MY, Myanmar; PH, Philippines; SI, Singapore; TH, Thailand; VI, Vietnam; AF, Afghanistan; BA, Bangladesh; BH, Bhutan; IND, India; MAL, Maldives; NE, Nepal; PA, Pakistan; SR, Sri Lanka.

rRNA and ZFX/ZFY (P1/P2) primers, 200 µM dNTPs, 0.5 U *Taq* polymerase (Fermantas) and 40–50 ng of each DNA sample in a 20 µl reaction volume. For the primers of Set I, the cycling included 40 cycles of denaturation at 95°C for 45 seconds, annealing at 55°C for 1 minute, extension at 72°C for 1 minute and a final extension cycle at 72°C for 20 minutes. For the primers of Set II and Set III, all the conditions were the same as those of Set I except that the primer concentration was 0.3 µM. The amplified PCR products were subjected to 2.5% agarose gel electrophoresis for physical identification. The gel was prepared using 1× TAE buffer (40 mMTris acetate, 1 mM EDTA, pH 8) containing 0.2 µg/mL EtBr, and photography was carried out under UV light in the gel documentation system. We identified the sex based on the presence or absence of Y-specific bands in the samples. In addition, after screening for positive amplification with all three sets of primers, a total of 218 samples (leopard, 53; otter, 93; goral, 31; chital, 13; sambar, 20; nilgai, 4; elephant, 2; tiger, 2) were also tested. The number of samples tested with each sex marker is provided against the respective primer set in table 2.

Validating the replicability of primers

To check the repeatability of co-amplified mitochondrial DNA as an internal primer for PCR amplification was also crosschecked using an X chromosome linked to a ZFX primer. After the primer set was successfully amplified for the 20 mammalian species, we also tested these primer sets with a larger number of samples (n = 218) of different mammalian species, the details of which are shown in table 2. Furthermore, the wide applicability of these markers have also been used to understand male-biased predation by tiger using single hair samples of four prey species obtained from tiger scat; these findings were published in a separate paper (De et al. 2018).

RESULTS

We tested the reliability of PCR-based sex identification assays using three sets of primers for 20 Indian mammalian species and suggested its applicability in mammals. The 44 samples from different mammalian species of known sexes were all amplified successfully with all three sets of primers, which had different amplicon sizes. The primers of Set I amplified one band of 12S rRNA (384 bp) and a second band, that of a sex-specific SRY primer (157 bp, Fig. 2; Set I). The upper band of P1/P2 (internal marker, 445 bp) and the lower band of SRY- Y53-3C and Y53-3D (225 bp) could be visualized (Fig. 2; Set II) using the primers of Set II. With the third set of primers (Set III), the lower band of 12s rRNA (151 bp) and the upper band of SRY- Y53-3C and Y53-3D (225 bp) were amplified (Fig. 2; Set III). In addition, after these primer sets were screened for amplification of all 20 mammalian species, they were used to identify the sex of different mammalian species using the different kind of samples (n = 218). The species tested are indicated against their respective primers (Table 2). We successfully extracted DNA from single hairs of tigers (n = 2), elephants (2), sambars (20), nilgais (4) and chitals (13) with the third set of primers. This set of primers has small amplicon sizes (151–225 bp) for both genes and can easily amplify DNA from degraded samples.

DISCUSSION

The method of PCR-based sex identification using three sets of primers described in the foregoing can be used for different mammalian species. It has potential use in identifying sex from small quantity samples, which is useful because samples in wildlife forensics and non-invasive genetics often have lower quality DNA. Such poor-quality DNA, especially with the presence of inhibitors, make obtaining large amplicons

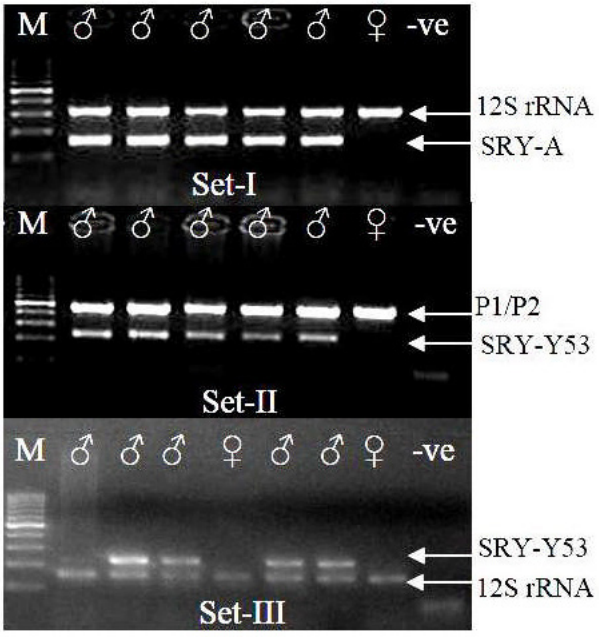

Fig. 2. PCR amplification of sex primer with three different sets of primers. ♂, male; ♀, female; +♂ and +♀, positive male and positive female; -ve, negative control; M, 100 bp leader. The samples amplified with Set-I and Set-II in figure 2 are identical; Set-III is depicted amplifying a different set of samples.

difficult.

Most wildlife forensics and endangered species samples collected during status surveys (including non-invasive genetic samples) yield low-quality DNA in small quantities. Set I and Set II are useful for identifying sex from a wide range of samples that are encountered in wildlife forensics, such as blood strains, tissues and tanned skins (Hsieh et al. 2006; Banks and Wright 2007; Caniglia et al. 2010; Jun et al. 2011). Set III has amplicons of small sizes, and hence it can be used with degraded samples, such as those from articles made with hair (paint and shaving brushes, also tested in this study) (Domingo-Roura et al. 2006) and tanned skin (clothes, purses, ties, belts, etc.). Set III can also be used with samples such as antlers, horns, ivory and bones, which are frequently encountered in the illegal trade market (Banks and Wright 2007; TRAFFIC 2011). This primer set also has a wide applicability in amplifying low-quality carnivore and herbivore DNA obtained from different samples (Farrell et al. 2000; Brinkman and Hundertmark 2009), especially in hair obtained from snares or prey species in carnivore scat (De et al. 2018); it has been proven that only a single hair is needed for DNA-based examination.

With molecular tools being used more and more in wildlife science, our study provides a method for low-cost molecular sexing of mammals in South and Southeast Asian countries using various biological samples. All three sets of primers may be used in wildlife forensic work and the conservation biology of South and Southeast Asian mammals, as most of the sample types yielded DNA < 500 bp long (Baker et al. 2001; Butler et al. 2003; Brinkman and Hundertmark 2009; Goyal et al. unpublished data). We suggest that at least two sets of primers be used for any biological samples to avoid any ambiguity.

CONCLUSIONS

Low-cost molecular sexing was performed on 20 mammalian species distributed in South and Southeastern countries. Three sets of universal primers were checked and successfully amplified in all the species, and they exhibited the potential to identify the sex of these species using the Y chromosome. Most samples utilized in conservation genetics are of poor quality and it is difficult to perform a single run amplification of larger fragments of DNA. Therefore, each set of primers was targeted based on the type of sample; the primers generated fragments of 151–445 bp long. The use of a combination of sex-linked genes and internal (sex-linked and mitochondrial) DNA primers provide unambiguous results for sex identification (Ortega et al. 2004). The method describes here has a wide applicability for conservation genetics and wildlife forensics in understanding sex-biased poaching and sex ratios in populations as a low-cost method. Among the different sets of primers used, Set III is the most effective for identifying sex using poor quality samples, such as single hairs from carnivore scats (De et al. 2018), and understanding biased sex ratios in carnivore diets.

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Authors' contributions: BDJ designed the study, performed DNA extraction and further laboratory procedures such as sequencing and data analysis, and wrote the MS; RD helped in laboratory procedures and co-wrote the MS. SPG conceptualized the idea, provided the laboratory support, supervised the work, edited and commented of the MS.

Competing interests: BDJ, RD and SPG declare that they have no conflict of interest.

Availability of data and materials: All required information are given in the Materials and Methods section.

Consent for publication: Not applicable.

Ethics approval consent to participate: No ethical permission required as all the samples used from the reference repository of Wildlife Institute of India.

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