RESEARCH ARTICLE

ILLEGAL HUNTING CASES DETECTED WITH MOLECULAR FORENSICS IN INDIA

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ARTICLE INFO	ABSTRACT
	Illegal hunting is one of the major threats to wildlife flora and fauna. In present study, we identified five cases of suspected wildlife poaching by using the molecular techniques. Mitochondrial cytochrome b (Cyt-b) and 12S ribosomal rRNA (12S rRNA) genes identified five wild species, Chinkara (*Gazella bennetti*), Peafowl (*Pavo cristatus*), Musk deer (*Moschus chrysogaster*), Black bear (*Selenarctos thibetanus*) and Tiger (*Panthera tigris*). In India, poaching and hunting is a illegal offense, and based on this evidence, the accused were found guilty and punished. The genetic analysis used in this investigative study was suitable to diagnose the species killed and solve these criminal investigations. Molecular DNA forensic techniques can provide an important tool that enables local law enforcement agencies to apprehend illegal poachers

INTRODUCTION

Illegal hunting is one of the major threats to vertebrate populations in the world. Approximately 10-20% of vertebrates and plants population were extinct in last few decades. According to Interpol (International Policing Organisation), the illegal trade of animals and plants, their by products is a growing global black market commerce estimated to be worth more than USD $20 billion per year (IUCN., 1996, 1997, 2000). Overexploitation by human threaten about one third of endangered species of vertebrates (Primack., R 1998). Human has been exploiting wildlife and natural resources from last decades for food, medicine, pleasure and profit but commercial exploitation in recent years a main reason of declining and extinction of Flora and Fauna (Adrian *et al*., 2011). Thus the conservation of wild species becomes a major task due to the illegal trading and poaching. Due to the conservation purpose and for the prevention of illegal poaching and trading many species are listed as being protected both at a national and international level. There are currently 175 countries including India which are members of the Convention for the International Trade in Endangered Species of Flora and Fauna (CITES) -an organization that oversees the movement of protected and endangered species across international borders (CITES., 1973, 2010).

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Each member country of this organization is responsible for the implementation of the Convention at a national level. National legislation has been enacted in many countries which are specifically aimed at the protection of species within their own country. In analyses of forensic sample like trace and degraded samples molecular tests based on mitochondrial DNA (mtDNA) are recommended (Parson *et al*., 2000). The cytochrome b gene and 12S rRNA of mtDNA has been found to be a powerful indicator for identifying the species with DNA analysis techniques (Budowle *et al*., 2003) also used in studies of molecular evolution (Kocher *et al*., 1989; Montegelard *et al*., 1997; Zehner *et al*., 1998).

Here, we reported an investigative study of five suspected offenses of wildlife poaching in India. In October 2013, Manipur forest department seized 10 bone samples (B01-B08) during routine checkup near the forest area (case-01) and suspected to be carnivore origin. In November 2013, two meat samples (M-01 and M-02) seized by Rajasthan forest department (case 2) and sent to us for identification. In January 2014, we received another meat sample (M-03) seized by Maharashtra forest department suspected to be goat origin and in February 2014, two independent cases (Case-04 and 05) that contain meat samples (M-04 and M-05) were seized by Uttrakahnd forest department from two different places without any certainty. All five seizures were performed in the central-western and north-east region of India, and species identifications were not possible from morphological data.

Therefore, to prove the criminal activity and to enable the appropriate law enforcement action these samples were processed to DNA analysis.

MATERIAL AND METHODS

DNA extraction, PCR amplification and sequencing Genomic DNA isolation from five meat samples (M01-M05) using the Qiagen DNeasy Tissue Kit (QIAGEN, Germany) following the manufacturer's recommendations protocol (incorporating an overnight digest) and bone samples (B01-B08) were processed with commercial available DNA extraction kit (GeNei™).

We amplified Cyt b gene of ca. 350 bp and 12S rRNA gene of ca 421 bp using universal primers (Meyer *et al*., 1995; Girish *et al*., 2004). All PCR reactions were carried out in Applied Biosystems® 3700 Thermal Cycler (ABI) with standard PCR cycling conditions (Meyer *et al*., 1995; Girish *et al*., 2004). The amplifications of both genes were carried out in a final volume of 25 µl containing 100 ng of DNA, 0.3 mM dNTPs, 1× PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 2.5 mM MgCl2, 2.5 U Taq DNA polymerase (Invitrogen) and 8 pmol of each primer. PCR products were purified using Exo-SAP to remove residual oligonucleotides and dNTPs prior to sequencing reaction. The forward and reverse primer were used independently for the sequencing reactions using the Big Dye® Terminator v3.1 Cycle Sequencing kit to generate sequence from both ends. The products were purified using a standard ethanol precipitation method and sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystems, USA).

Data analysis

Individual Cyt b and 12S rRNA sequences were cleaned and validated using SEQUENCHER 4.8 (Gene Codes Corporation, Ann Arbor, MI). Multiple sequence alignments were performed using the CLUSTAL W algorithm implemented in BIOEDIT version 7.0.5.3 (Hall TA., 1999). The sequences obtained from the unknown skin specimens were compared with the sequences publicly available at GenBank using BLAST search tool of NCBI (http://blast.ncbi.nlm.nih.gov/). All the sequences that show similarity with the unknown sequence were downloaded (Table 1) and used for phylogenetic analysis using Kimura 2 parameter distance matrix with the neighbor-joining method as implemented in Mega v5.0 software (Tamura *et al*., 2011).

RESULTS AND DISCUSSION

All meat samples (M01-M04) yielded moderate quality of genomic DNA while bone samples (B01 to B08) did not yield visible genomic DNA on gel. However, all samples yielded high quality sequences. The sequences obtained were submitted as independent entries in BLAST search for the most similar sequences using the default Mega blast algorithm parameters. The sequences of the seized samples were compared against those of species that were likely to be hunted or consumed at the seizure region (Table 1), which were downloaded from GenBank and WII reference sequences of different species those are highly in wildlife trades. The Cyt b and 12S rRNA sequence of used seizers produced readable sequences of 350 bp and 420 bp.

Table 1. Details of used species with GenBank Accretions number of used Cytochrome b and 12S ribosomal RNA

Species/SID	Common name	GenBank Accession number	
		Cytb	12S rRNA
Mammals			
Axis axis/AA	Chital	JN093090	JN093077
Axis porcinus/AP	Hog deer	EF579805	AY775785
Rusa unicolor/RU	Sambar	JN861032	GQ463698
Rucervus duvaucelii/RD	Swamp deer	EF079 830	EU908275
Moschus chrysogaster/MC	Musk deer	NC020093	AY184426
Muntiacus muntjak/MM	Barking deer	JQ991600	AF294731
Antelope cervicapra/AC	Black buck	AF022058	AP003422
Tetracerus quadricornis/TQ	Four horned antelope	AF036274	EF175739
Boselaphus tragocamelus/BT	Nilgai	EF536350	M86494
Capricornis sumatraensis/CS	Serow	FJ207534	AY670660
Gazella bennetti/GB	Chinkara	N410357	EF133853
Sus scrofa/SS	Wild pig	KC505411	KC505411
Sus scrofa domestica/SSD	Domestic pig	NC012095	NC012095
Melursus ursinus/MU	*Sloth Bear*	EF196662	EF196662
Selenarctos thibetanus/ST	*Black bear*	EU264545	FM177759
Panthera tigris/PT	Tiger	KC879297	AY736583
Canis aureus/CA	Jackal	AF028138	DQ102371
Panthera pardus/PD	Leopard	JF720185	AM779888
Birds			
Pavo cristatus/PC	Peafowl	AY078585	AY952766
Otis tarda tarda /OTT	Great Bustard	AY078585	NA
Chlamydotis undulate/CU	Houbara Bustard	AJ511450	NA
Gallus gallus/GG	Chicken	EF362711.	EF362711
Coturnix Coturnix /CC	Quail	EU839461	AM902516
Francolinus gularis/FG	Swamp Francolin	FGU90649	NA
Gyps indicus/GI	Long-billed Vulture	EU496442	NA
Catreus wallichi/CW	Cheer Pheasant	AF028792	KC749451

BLAST analysis of Cyt b indicated that the M-01 and M-02 was highly similar (100%) to the Chinkara (*Gazella bennetti*) with reference sequence, the M-03 was similar (100%) to Peafowl (*Pavo cristatus*), the M-04 was similar (100%) with musk deer (*Moschus chrysogaster*), the sample M-05 was similar (100%) with Black bear (*Selenarctos thibetanus*) and the all bone samples B01 to B08 were highly similar (100%) to Tiger (*Panthera tigris*). Similar result is found in the 12S rRNA gene (Table 2, 3).

The neighbor-joining phylogenetic tree showed that BLAST analysis and neighbor-joining boot strap value are similar in all identified species in Cytochrome b gene but in 12S rRNA gene neighbor-joining boot strap value are different in three seizers (M-01, M-02 and M-04) showed 99% similarity, all bone sample showed 99% similarity and remaining two sample M-03 and M-05 similar with Cyt b result (100%) (Fig 1 and Fig 2). The Species specific polymorphic sites in Cyt b and 12S rRNA mitochondrial gene are given in Table 4. A and 4.B.

Table 2. Similarities in the Cytb locus between cases samples and most similar species available in Gen Bank

Specimen	Species with the highest similarity (GenBank accession in Table 1.)	Query coverage (%)	Similarity (%)
M0I-02	*Gazella bennetti*/GB	100.0	100.0
M-03	*Pavo cristatus*/PC	100.0	100.0
M-04	Moschus chrysogaster/MC	100.0	100.0
M-05	*Selenarctos thibetanus*/ST	100.0	100.0
B01-10	*Panthera tigris*/PT	100.0	100.0

Table 3. Similarities in the 12S rRNA locus between cases samples and most similar species available in GenBank

Specimen	Species with the highest similarity (GenBank accession in Table 1.)	Query coverage (%)	Similarity (%)
M0I-02	*Gazella bennetti*/GB	100.0	100.0
M-03	*Pavo cristatus* /PC	100.0	100.0
M-04	Moschus chrysogaster/MC	100.0	100.0
M-05	*Selenarctos thibetanus*/ST	100.0	100.0
B01-10	*Panthera tigris*/PT	100.0	100.0

Table 4.A. Cytochrome b Variable site within the identified species with case samples.B.12S rRNA Variable site within the identified species with case samples Table 4.A

Nt position	129	132	139	145	157	176	179	203	226	233	240	241	242	307	337
GU249571.1	A	A	C	C	A	A	A	C	A	A	C	G	A	A	C
GB	•	•	A	T	C	•	•	•	C	•	•	•	•	•	•
M-01	•	•	A	T	C	•	•	•	C	•	•	•	•	•	•
M-02	•	•	A	T	C	•	•	•	C	•	•	•	•	•	•
PC	T	•	•	•	•	•	•	•	•	•	•	•	•	C	A
M-03	T	•	•	•	•	•	•	•	•	•	•	•	•	C	A
MC	C	•	A	•	•	•	•	T	•	•	•	•	•	•	•
M-04	C	•	A	•	•	•	•	T	•	•	•	•	•	•	•
ST	•	G	G	•	T	T	G	•	•	•	T	C	G	G	•
M-05	•	G	G	•	T	T	G	•	•	•	T	C	G	G	•
PT	•	•	•	•	C	•	•	•	•	G	•	•	•	•	T
B-01	•	•	•	•	C	•	•	•	•	G	•	•	•	•	T
B-02	•	•	•	•	C	•	•	•	•	G	•	•	•	•	T
B-03	•	•	•	•	C	•	•	•	•	G	•	•	•	•	T
B-04	•	•	•	•	C	•	•	•	•	G	•	•	•	•	T
B-05	•	•	•	•	C	•	•	•	•	G	•	•	•	•	T
B-06	•	•	•	•	C	•	•	•	•	G	•	•	•	•	T
B-07	•	•	•	•	C	•	•	•	•	G	•	•	•	•	T
B-08	•	•	•	•	C	•	•	•	•	G	•	•	•	•	T

Table 4.B

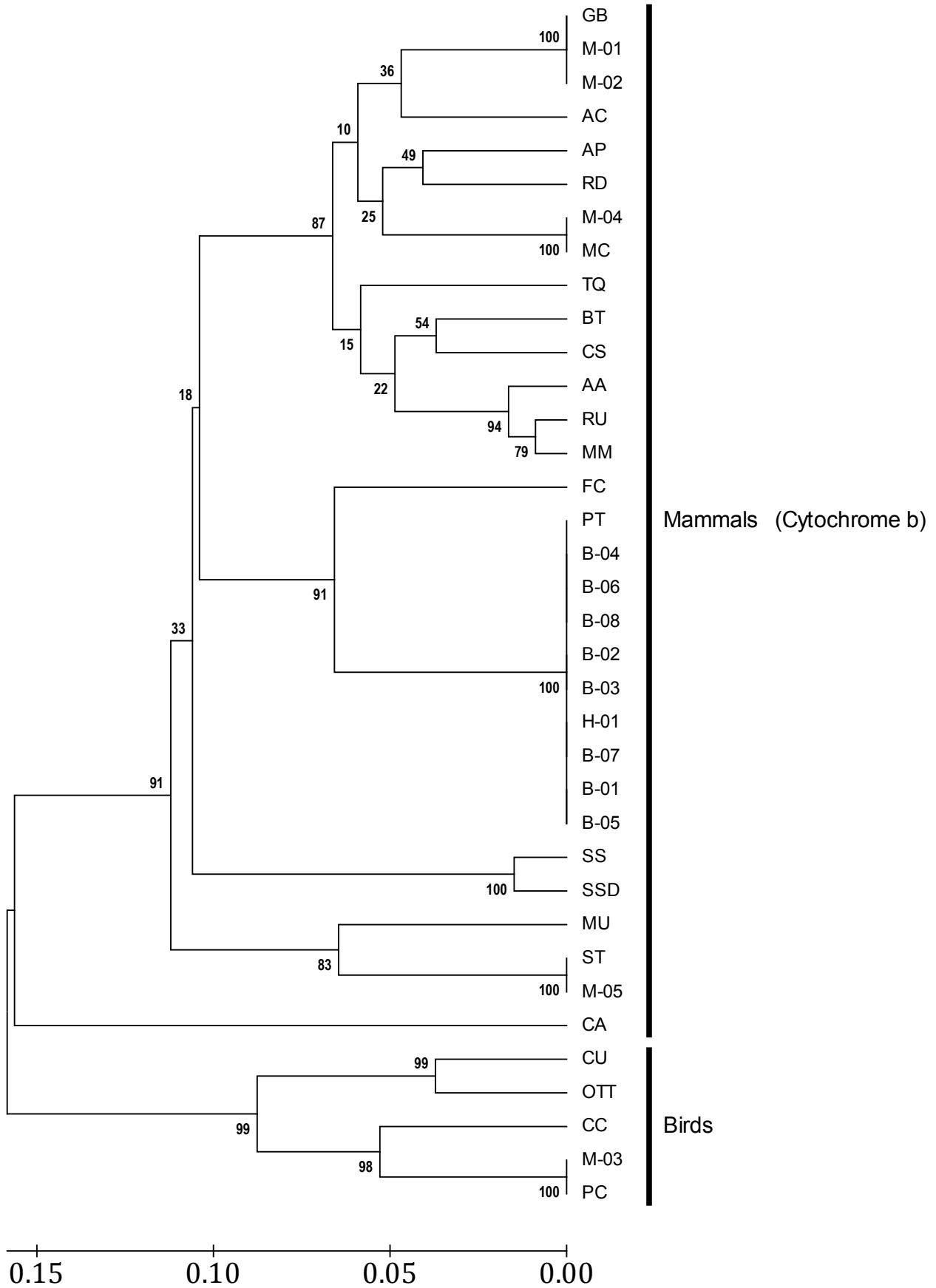| Nt position	778	851	897	925	973	1013	1055	1128
GU249571.1	A	A	C	A	T	A	A	A
GB	C	T	A	•	A	•	•	•
M-01	C	T	A	•	A	•	•	•
M-02	C	T	A	•	A	•	•	•
PC	•	T	A	T	C	•	G	C
M-03	•	T	A	T	C	•	G	C
MC	•	T	C	G	A	•	•	•
M-04	•	T	C	G	A	•	•	•
ST	T	T	A	G	A	•	•	G
M-05	T	T	A	G	A	•	•	G
PT	•	C	T	•	A	T	•	G
B-01	•	C	T	•	A	T	•	G
B-02	•	C	T	•	A	T	•	G
B-03	•	C	T	•	A	T	•	G
B-04	•	C	T	•	A	T	•	G
B-05	•	C	T	•	A	T	•	G
B-06	•	C	T	•	A	T	•	G
B-07	•	C	T	•	A	T	•	G
B-08	•	C	T	•	A	T	•	G

Figure 1. Cytochrome b Neighbour-joining phylogenetic tree showing the relationships of Seizure with the other species
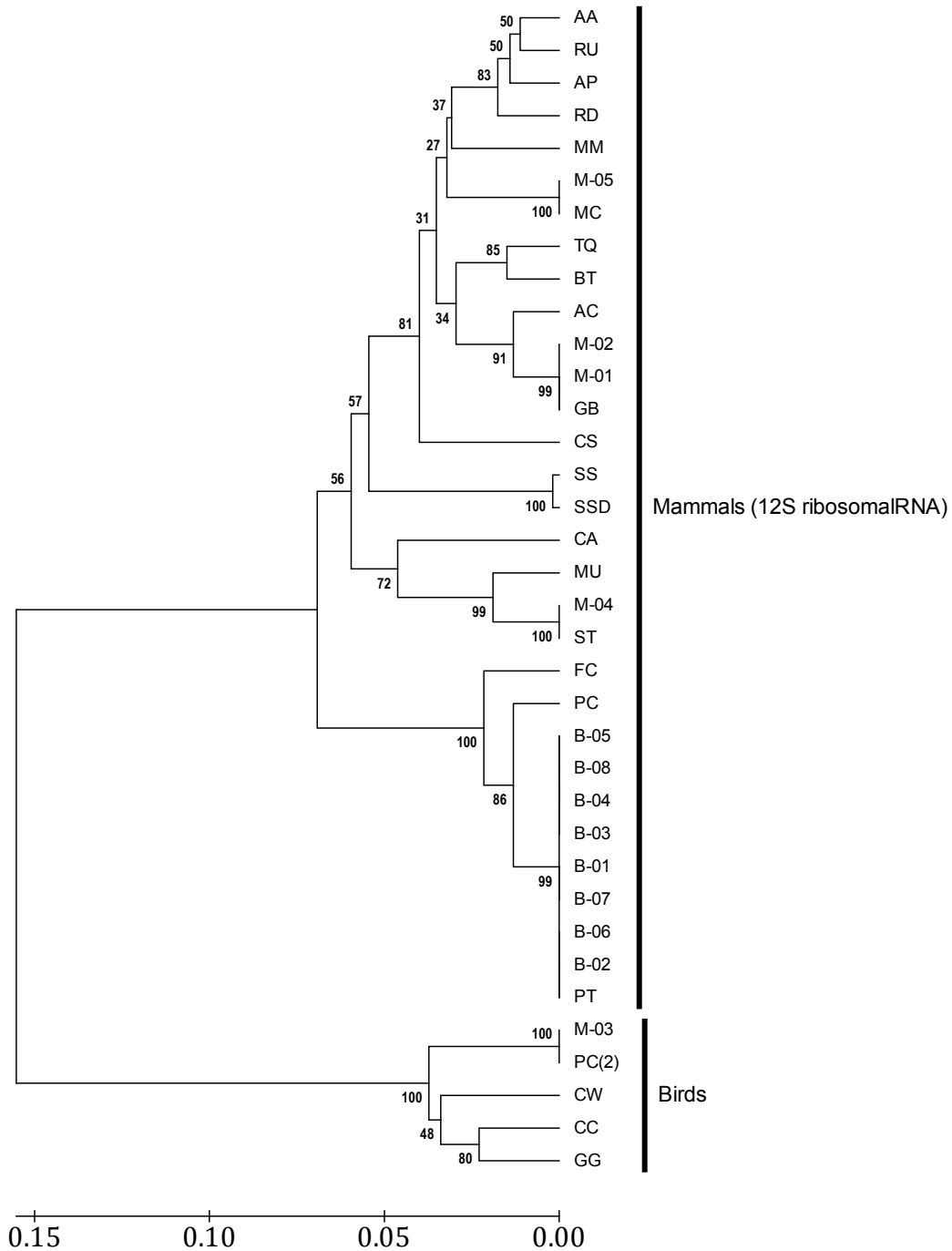
Figure 2. 12S rRNA Neighbor-joining phylogenetic tree showing the relationships of Seizure with the other species

Conclusion

In conclusion, we here describe the need of proper planning to the wildlife species conservation especially to endangered species like Tiger, black bear and musk deer the utility of Cyt b and 12S rRNA mitochondrial gene sequence analysis in the species identification of wildlife offences. This result shows the cytochrome b gene is the most robust and good mitochondrial gene to see inter and intra differences within and between the species with compare to 12S rRNA to solving the forensic cases based on DNA analysis. The purpose of this study to give contribution in the conservation of the wildlife fauna and on the basis of molecular study we prepared forensic sample DNA database which contributes to reticence for change. These database developed to help investigate futures wildlife crime and have been standardized a protocol to solve cases in wildlife forensic.DNA database may not have exploited fully and could provide lead to new investigative questions.

Acknowledgment

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