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Wildlife Forensic Case Study for Identification of Species from Pangolin Scales Using Mitochondrial DNA

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Abstract

Pangolins are considered the most traded mammals worldwide. In India, the species found are Indian and Chinese pangolins (Manis crassicaduata and Manis pentadactyla) which are protected under the ambit of the Wildlife (Protection) Act, 1972- Schedule I and CITES- Appendix I. Pangolins are poached for their scales which are deemed to possess traditional medicinal properties and for meat, usually consumed as a delicacy. Analysis of mitochondrial DNA can be used for species identification from pangolin scales, to provide evidence of illegal wildlife trade. Eight sub-samples from a single seized consignment containing suspected pangolin scales were processed for DNA analysis by amplifying and sequencing partial fragments of mitochondrial genes Cytochrome b and 12S rRNA. Sequences generated from all 8 sub-samples matched with Manis crassicaudata sequences in GenBank database with a percentage identity of 99-100%. Based on evidence of percentage identity, genetic distance and maximum-likelihood phylogenetic trees of the two mtDNA genes, seized material was concluded to be from Indian pangolin (Manis crassicaudata).The study demonstrates the utility of DNA analysis using mitochondrial DNA markers for the identification of species from keratinized products (such as scales)and can serve as evidence of illegal trade of wildlife products for prosecuting with reference to the Indian Wildlife (Protection) Act, 1972.

Keywords: DNA analysis, mitochondrial DNA, CITES, WPA, illegal wildlife trade, pangolin scales.

Introduction

Pangolins, considered the most traded mammal worldwide belong to the family Manidae and order Pholidata (Weber 1904)[1]. Totally comprised of 8 species of which 4 are native to Africa (ground-dwelling and arboreal) and 4 native to Asia (ground-dwelling), pangolins are Evolutionarily Distinct and Globally Endangered (EDGE) mammals with significant ecological roles[2,3]. The enigmatic mammals inhabit the African and Asian tropical and inter-tropical zones[4]. Increasing changes in habitat triggered by anthropogenic activities as well as the rise of illegal wildlife trade are major threats to the survival of pangolins worldwide. The population size of all the 8 extant species has drastically declined, with a higher extinction risk being posed to the Asian species[5]. Slow growth rates coupled with the low reproductive recovery rates in impacted areas make pangolins more vulnerable to extinction[6]. In India, both Indian (*Manis crassicaudata)* and Chinese (*Manis pentadactyla*) pangolins are distributed, and theyare conferred highest protection under Schedule I of the Wildlife (Protection) Act (WPA), 1972 of India[7]. Pangolins are illegally traded for bushmeat and scales in the African and Asian continents, with a huge rise in demand, smuggling and illegal trade of their products over the past three decades despite the ban of international trade of all 8 pangolin species and their derivatives under the Appendix I of CITES[8]. Unsustainable and unregulated illegal trade of pangolin products has long been driven by demands from the traditional medicinal use of scales as well as meat for consumption as a delicacy[1]. Pangolin products widely traded in the illegal market include scales, scale powder, meat, smoked carcasses and even embryos. Identification of processed scales traded in the markets is challenging as it is complicated by the introduction of counterfeited scales from other species, such as hoof nails from sheep (*Ova aries*), cattle (*Bos taurus*) and pigs (*Sus scrofa and Sus scrofa domestica*)[8]. Accurate species identification from seized suspected pangolin products is therefore extremely crucial for conservation of pangolins and for curbing their illegal trade. Use of reliable methods for identification of species is very important in providing evidence to forensic cases where processed animal parts such as pangolin scales are confiscated[5]. Following the confiscation of pangolin scales, one of the main challenges is to ascertain the species identity. Visual identification of pangolin scales can be challenging owing to the presence of limited morphological features, thereby raising the need for DNA based analysis[9]. Mitochondrial DNA (mtDNA) markers such as *Cytochrome b* (Cytb) and *12S rRNA* are used to compare species of the same genera or family owing to high copy number per cell, high rates of substitution, maternal inheritance and absence of recombination[2]. Our case report describes amplification of Cytb and 12S rRNA regions of mtDNA from pangolin scales for accurate identification of species for providing evidence of wildlife products being illegally traded to prosecute with reference to the Indian Wildlife (Protection) Act, 1972.

Materials and methods

Laboratory processing of seized sample: Consignment seized at Tamil Nadu by the State Forest Department, containing suspected pangolin scales was referred to Advanced Institute for Wildlife Conservation for species identification. From the consignment, 8 sub-samples consisting of powder from the suspected pangolin scales were processed for DNA analysis by drilling through the internal surface that was connected to the skin using Bosch impact drill (GSB 10 RE). Powder obtained from drilling was collected in 2mL centrifuge tubes and impurities were removed by washing twice with sterile distilled water. Extraction of DNA was performed using Qiagen DNeasy Blood and Tissue kit (Qiagen, Germany) as per manufacturer's protocols, with 16 hour incubation period for digestion. Quality and quantity of DNA was measured using the Nanodrop One (Thermo Scientific) spectrophotometer using 1µL of sample. Extracted DNA was stored at (-20˚C) until use for amplification.

Amplification by PCR and sequencing: Partial fragments of Cytb (350bp) and 12S rRNA (450bp) regions were amplified using Eppendorf Nexus GSX1 Master cycler at the reaction conditions of initial denaturation for 5 min. at 95˚C, after which followed 30 seconds of denaturation at 95˚C, 30 seconds of annealing at 55˚C, 45 seconds of extension at 72˚C for 35 cycles and final extension at 72˚C for 10 min[10]. Total reaction volume of 10µL was composed of 1X Taq Buffer (KAPA Biosystems, SIGMA), with dNTPs at a concentration of 0.25mM, both forward and reverse primer each of 0.4 µM, 2.5mM $MgCl_2$, 0.25 U Taq DNA Polymerase (KAPA Biosystems, SIGMA) and template DNA of concentration 2-10ng/µL. To prevent chances of contamination, negative controls were included in every DNA extraction step and the PCR reactions were performed with positive as well as non-template controls. The PCR products were subjected to purification using QIA quick Gel Extraction Kit and sequenced bidirectionally using ABI 3730 DNA analyzer (Applied Biosystems, USA).

Sequence analysis: Forward and reverse sequences obtained were processed and assembled using BioEdit[11]. The NCBI BLAST tool was used to identify percentage identity of query sequence with the GenBank database sequences[12]. The query Cytb and 12S rRNA sequences were subjected to alignment with the most homologous sequences (with a threshold of ≥90% identity) and African pangolin sequences downloaded from GenBank database. Maximum-likelihood trees using Kimura-2-parameter model were reconstructed with 1000 bootstrap replicates and Tamura-Nei model was used to construct genetic distance matrices of the two genes in MEGA X[13-15].

Results and discussion

Concentration of genomic DNA isolated from all 8 sub-samples ranged from 2-10ng/µL. Positive PCR amplification and good quality sanger sequencing results were obtained for the 8 sub-samples using the Cytb and 12S rRNA primers. No cross amplification was observed in negative DNA extraction and PCR amplification controls. Using NCBI BLAST tool, sequenced products matched with *Manis crassicaudata* (Indian pangolin) sequences in the GenBank database, with a percentage identity of 99-100%. The Cytb and 12S rRNA sequences generated were deposited to GenBank database with accession numbers as recorded in Table-1.

Table-1: Similarities of Cytb and 12S rRNA sequences of sub-samples 1-8 based on NCBI-GenBank BLAST search.

Query sample	Species with highest similarity	Cytb		12S rRNA	
		Percentage Identity (%)	GenBank Accession No.	Percentage Identity (%)	GenBank Accession No.
Sub-sample 1	*Manis crassicaudata*	100	OK018348	100	MZ983632
Sub-sample 2	*Manis crassicaudata*	100	OM032526	100	OM022007
Sub-sample 3	*Manis crassicaudata*	100	OL901595	100	OL742109
Sub-sample 4	*Manis crassicaudata*	100	OL901598	100	OL742110
Sub-sample 5	*Manis crassicaudata*	100	OL901596	99.75	OL889911
Sub-sample 6	*Manis crassicaudata*	100	OL901597	100	OL889915
Sub-sample 7	*Manis crassicaudata*	100	OL901599	100	OL889913
Sub-sample 8	*Manis crassicaudata*	100	OL901600	100	OL889912

Genetic distance matrix was constructed using homologous sequences from the BLAST search as well as from GenBank database. The genetic distance matrix for Cytb and 12S rRNA sequences of the sub-samples showed no genetic distance with the GenBank reference sequences of *Manis crassicaudata* (Table-2 and Table-3). Based on the matrix, Cytb sequences of the 8 sub-samples varied from reference sequences by 0-0.8% while the 12S rRNA sequences varied from reference sequences by 0-0.3%. Maximum-likelihood phylogenetic trees for both the genes correlated with the BLAST and genetic distance indices, with the query sequence being classified under the same clade as that of Indian Pangolin with greater than 70% bootstrap support (Figure-1 and Figure-2). Based on the data analysed with regard to percentage identity, genetic distance and phylogenetic trees of the two mtDNA gene sequences obtained from all the 8 sub-samples, it has been concluded that the seized material is from *Manis crassicaudata*.

Determination of species from scales processed for medicinal purposes can be challenging as the scales are treated at high temperatures leading to degradation of DNA[8]. In this case report, species level identification was achieved by analysis of mtDNA[5,16,17]. The *COI* gene has been used to resolve pangolin phylogeny and has proven efficacious in delineating *M. pentadactyla* samples to multiple geographic origins using reference scale samples collected from known geographic locations of Nepal[5,18,19]. Use of *COI*, *Cytb* and D-loop gene regions for reliable and accurate species identification of all African pangolin species was reported[5]. Mitochondrial D-loop region based species identification from scales has also been reported[9]. Reports have demonstrated use of different combinations of mtDNA genes *Cytb, 12S rRNA, 16S rRNA* and *COI* for species identification of pangolins products, such as *Cytb* and *16S rRNA*, *Cytb* and *COI*[8,16,20].

Table-2: Genetic Distance Matrix computed using Tamura-Nei model in MEGA-X for Cytb gene.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Sub-sample 1	***																									
Sub-sample 2	0.000	***																								
Sub-sample 3	0.000	0.000	***																							
Sub-sample 4	0.000	0.000	0.000	***																						
Sub-sample 5	0.000	0.000	0.000	0.000	***																					
Sub-sample 6	0.000	0.000	0.000	0.000	0.000	***																				
Sub-sample 7	0.000	0.000	0.000	0.000	0.000	0.000	***																			
Sub-sample 8	0.000	0.000	0.000	0.000	0.000	0.000	0.000	***																		
MG196305 Manis crassicaudata Cytb	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.008	***																	
MG196304 Manis crassicaudata Cytb	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.008	***																
MT796332 Manis crassicaudata Cytb	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.008	0.000	***															
MG196306 Manis crassicaudata Cytb	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.012	0.004	0.004	***														
MN365835 Manis javanica Cytb	0.107	0.107	0.107	0.107	0.107	0.107	0.107	0.107	0.107	0.107	0.107	0.107	***													
MN365836 Manis javanica Cytb	0.097	0.097	0.097	0.097	0.097	0.097	0.097	0.097	0.096	0.097	0.097	0.101	0.040	***												
MT796325 Manis pentadactyla Cytb	0.098	0.098	0.098	0.098	0.098	0.098	0.098	0.098	0.107	0.098	0.098	0.102	0.142	0.132	***											
MN365834 Manis pentadactyla Cytb	0.093	0.093	0.093	0.093	0.093	0.093	0.093	0.093	0.102	0.093	0.093	0.097	0.148	0.127	0.004	***										
MG196299 Phatiginus tetradactyla Cytb	0.239	0.239	0.239	0.239	0.239	0.239	0.239	0.239	0.251	0.239	0.239	0.244	0.246	0.254	0.217	0.224	***									
NC_004027 Phatiginus tetradactyla Cytb	0.220	0.220	0.220	0.220	0.220	0.220	0.220	0.220	0.209	0.220	0.220	0.225	0.231	0.243	0.246	0.253	0.193	***								
MT875193 Smutsia temminckii Cytb	0.226	0.226	0.226	0.226	0.226	0.226	0.226	0.226	0.226	0.226	0.226	0.232	0.226	0.243	0.230	0.236	0.167	0.187	***							
NC_025769 Smutsia temminckii Cytb	0.215	0.215	0.215	0.215	0.215	0.215	0.215	0.215	0.227	0.215	0.215	0.220	0.210	0.232	0.222	0.229	0.134	0.202	0.045	***						
MG196310 Phatiginus tricuspis Cytb	0.209	0.209	0.209	0.209	0.209	0.209	0.209	0.209	0.208	0.209	0.209	0.214	0.233	0.232	0.235	0.228	0.189	0.104	0.229	0.259	***					
NC_026780 Phatiginus tricuspis Cytb	0.202	0.202	0.202	0.202	0.202	0.202	0.202	0.202	0.202	0.202	0.202	0.207	0.224	0.224	0.237	0.230	0.164	0.094	0.210	0.226	0.062	***				
MT875191 Smutsia gigantea Cytb	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.213	0.209	0.230	0.231	0.238	0.144	0.136	0.105	0.115	0.182	0.156	***			
NC_036064 Smutsia gigantea Cytb	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.213	0.209	0.230	0.231	0.238	0.144	0.136	0.105	0.115	0.182	0.156	0.000	***		
MG196308 Manis culionensis Cytb	0.115	0.115	0.115	0.115	0.115	0.115	0.115	0.115	0.114	0.115	0.115	0.120	0.052	0.048	0.146	0.146	0.267	0.252	0.236	0.229	0.241	0.233	0.234	0.234	***	
NC_036434 Manis culionensis Cytb	0.115	0.115	0.115	0.115	0.115	0.115	0.115	0.115	0.114	0.115	0.115	0.120	0.052	0.048	0.146	0.146	0.267	0.252	0.236	0.229	0.241	0.233	0.234	0.234	0.000	***

Table-3: Genetic Distance Matrix computed using Tamura-Nei model in MEGA-X for 12S rRNA gene.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Sub-sample 1	***																									
Sub-sample 2	0.000	***																								
Sub-sample 3	0.000	0.000	***																							
Sub-sample 4	0.000	0.000	0.000	***																						
Sub-sample 5	0.003	0.003	0.003	0.003	***																					
Sub-sample 6	0.000	0.000	0.000	0.000	0.003	***																				
Sub-sample 7	0.000	0.000	0.000	0.000	0.003	0.000	***																			
Sub-sample 8	0.000	0.000	0.000	0.000	0.003	0.000	0.000	***																		
MG196304 Manis crassicaudata 12S	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	***																	
MG767207 Manis crassicaudata 12S	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	***																
NC_036433 Manis crassicaudata 12S	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.000	***															
MG767210 Manis crassicaudata 12S	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.000	0.000	***														
MG196309 Manis javanica 12S	0.047	0.047	0.047	0.047	0.050	0.047	0.047	0.047	0.047	0.047	0.047	0.047	***													
MN365836 Manis javanica 12S	0.047	0.047	0.047	0.047	0.051	0.047	0.047	0.047	0.047	0.047	0.047	0.047	0.013	***												
AY012154 Manis pentadactyla 12S	0.055	0.055	0.055	0.055	0.058	0.055	0.055	0.055	0.055	0.055	0.055	0.055	0.077	0.084	***											
MH423734 Manis pentadactyla 12S	0.051	0.051	0.051	0.051	0.054	0.051	0.051	0.051	0.051	0.051	0.051	0.051	0.065	0.072	0.016	***										
MF536687 Smutsia temminckii 12S	0.158	0.158	0.158	0.158	0.162	0.158	0.158	0.158	0.158	0.158	0.158	0.158	0.161	0.161	0.110	0.118	***									
NC_025769 Smutsia temminckii 12S	0.158	0.158	0.158	0.158	0.162	0.158	0.158	0.158	0.158	0.158	0.158	0.158	0.161	0.161	0.110	0.118	0.000	***								
MF536684 Smutsia gigantea 12S	0.157	0.157	0.157	0.157	0.161	0.157	0.157	0.157	0.157	0.157	0.157	0.157	0.157	0.165	0.127	0.123	0.062	0.062	***							
MG196303 Smutsia gigantea 12S	0.157	0.157	0.157	0.157	0.161	0.157	0.157	0.157	0.157	0.157	0.157	0.157	0.157	0.165	0.127	0.123	0.062	0.062	0.000	***						
KJ192567 Phatiginus tricuspis 12S	0.171	0.171	0.171	0.171	0.175	0.171	0.171	0.171	0.171	0.171	0.171	0.171	0.176	0.184	0.135	0.122	0.090	0.090	0.098	0.098	***					
MF536683 Phatiginus tricuspis 12S	0.165	0.165	0.165	0.165	0.169	0.165	0.165	0.165	0.165	0.165	0.165	0.165	0.170	0.178	0.130	0.117	0.102	0.102	0.094	0.094	0.016	***				
MF509825 Phatiginus tetradactyla 12S	0.181	0.181	0.181	0.181	0.185	0.181	0.181	0.181	0.181	0.181	0.181	0.181	0.187	0.195	0.143	0.143	0.103	0.103	0.106	0.106	0.033	0.037	***			
MG196299 Phatiginus tetradactyla 12S	0.187	0.187	0.187	0.187	0.191	0.187	0.187	0.187	0.187	0.187	0.187	0.187	0.192	0.200	0.148	0.148	0.107	0.107	0.110	0.110	0.037	0.040	0.010	***		
NC_036434 Manis culionensis 12S	0.033	0.033	0.033	0.033	0.037	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.013	0.013	0.070	0.058	0.164	0.164	0.161	0.161	0.180	0.174	0.191	0.196	***	
MG196308 Manis culionensis 12S	0.033	0.033	0.033	0.033	0.037	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.013	0.013	0.070	0.058	0.164	0.164	0.161	0.161	0.180	0.174	0.191	0.196	0.000	***

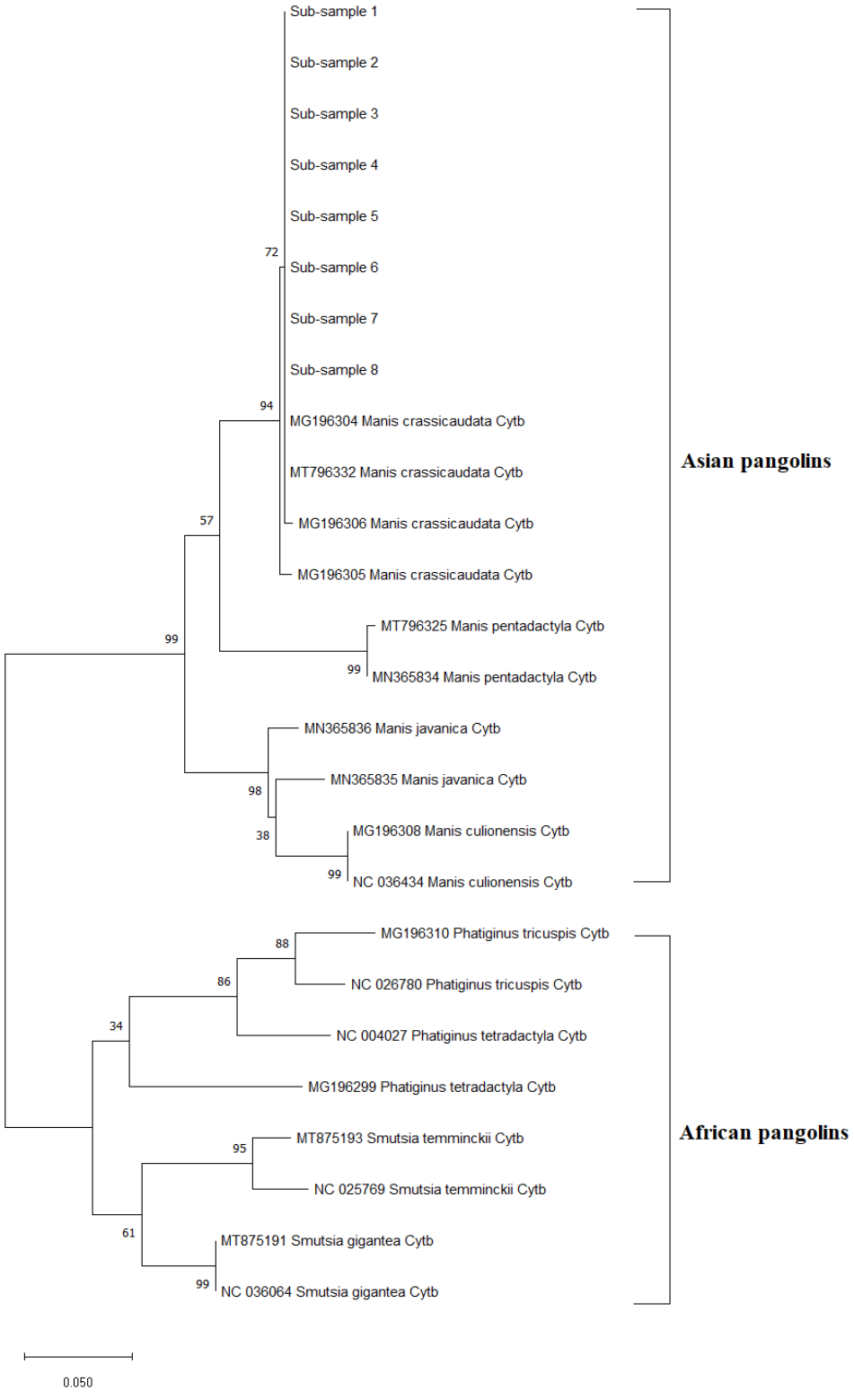

Figure-1: Reconstruction of Maximum-likelihood tree using Kimura-2-parameter model with 1000 bootstrap replicates for *Cytb* gene.

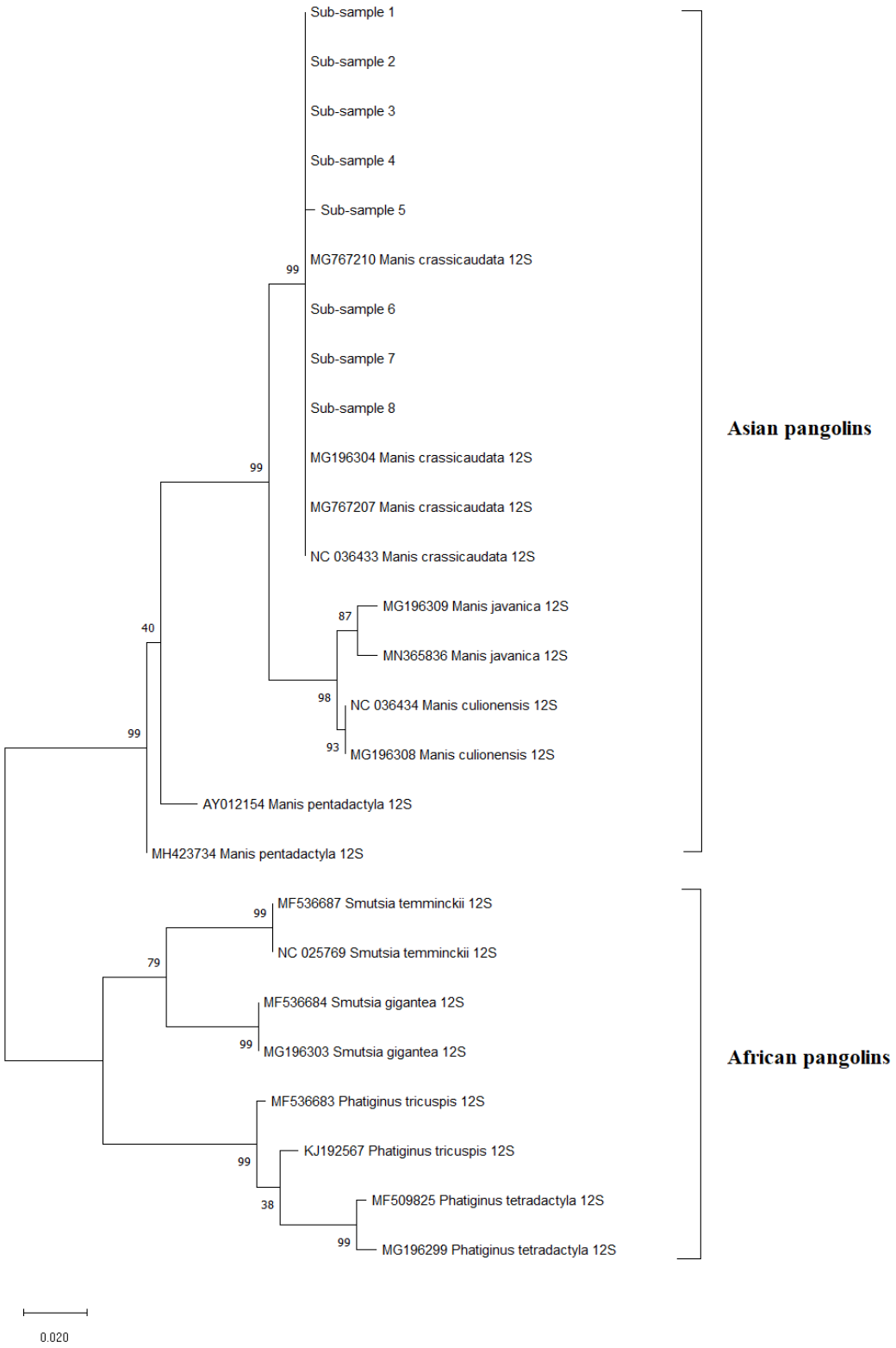

Figure-2: Reconstruction of Maximum-likelihood tree using Kimura-2-parameter model with 1000 bootstrap replicates for *12S rRNA* gene.

Conclusion

The combination of two genes used in this study, Cytb and 12S rRNA helped to effectively discriminate between the different pangolin species and arrive at a conclusion using DNA based evidence. Further utilization of DNA based techniques includes identification of geographic origin of the consignment, coupling the data with that of shipment details, place of seizure of consignment and final intended destination to provide significant information for identification of pangolin poaching hotspots targeted by illegal trade networks[17,21]. However, assignment of geographic provenance would require additional genetic tools and reference data on pangolin populations using single nucleotide polymorphisms (SNPs) which are known to be population level markers[5]. With Asian pangolins becoming rare due to their over-exploitation for illegal wildlife trade, several recent seizures have indicated that traffickers now resort to sourcing pangolin scales from Africa. Determination of the most appropriate genetic markers to trace the trade of pangolins with molecular tools could thus elucidate the potential of molecular methods in conservation of species that are on the brink of extinction[22]. Individualization of pangolin seizures can also be carried out using molecular studies to provide realistic figures of pangolins killed to serve as corroborative evidence in prosecution and contribute to standard operating protocols in handling pangolin scale seizures as demonstrated by[1].

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