**The forensic application of DNA barcoding for identification
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The forensic application of DNA barcoding for identification of illegally traded African pangolin scales

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Abstract

The escalating growth in illegal wildlife trade (IWT) and anthropogenic habitat changes threaten the survival of pangolin species worldwide. All eight extant species have experienced drastic population size reductions globally with a high extinction risk in Asia. Consequently, forensic services have become critical for law enforcement, with a need for standardised and validated genetics methods for reliable identifications. The seizure of three tonnes of pangolin scales, believed to have originated from Africa, by Hong Kong Customs Authorities provided an opportunity for the application of DNA barcoding in identifying scales. Three mitochondrial DNA gene regions (COI, Cyt *b* and D-loop) were amplified for a sub-sample of the confiscation, and compared to taxonomically verified references. All African four species were recovered as monophyletic with high interspecific uncorrected p-distance estimates (0.048-0.188) among genes. However, only three out of four African species (*Phataginus tricuspis*, *P. tetradactyla* and *Smutsia gigantea*) originating from West and Central Africa and one of the four Asian species (*M. javanica*) from Southeast Asia among scales were identified. Although the assignment of unknown scales to specific species was reliable, additional genetic tools and representative reference material is required in order to determine geographic origins of pangolin confiscations.

Keywords

Illegal Wildlife Trade; DNA barcoding; Forensic genetics; Pangolins; Pangolin scale confiscation; *Smutsia*; *Manis*

Introduction

The illegal poaching and trade of wildlife is a major biodiversity challenge that has contributed to a significant decline in populations of several species in Africa (Challender et al. 2015b). An increase in illegal wildlife trade (IWT) has been documented for all species of pangolin (Family: Manidae) that are exploited for bush meat, and body parts and scales, which have superstitious value and use in traditional medicine in Africa (*muthi*) and East Asia (Newton et al. 2008, Boakye et al. 2015, Zhang et al. 2015, Nijman et al. 2016). The IWT has been estimated to be worth US\$2.5 billion a year in East Asia and the Pacific, with pangolins contributing an estimated US\$100–150 million in Asia-Pacific (Zhang et al. 2015, Nijman et al. 2016). Therefore, illegal pangolin trade has continued to escalate, with pangolins now being the most trafficked wild mammal species by numbers (>10,000 individuals per year) globally (Davis 2014, Challender et al. 2015a). Furthermore, anthropogenic threats such as agricultural intensification have resulted in pangolin declines due to habitat loss and fragmentation (IUCN 2015). Particularly in South Africa, pangolins are electrocuted by electric fencing used on game and livestock farms (Bräutigam et al. 1994, Pietersen et al. 2014a). Pangolins are also considered to be highly vulnerable to extinction, due to their slow growth rates and low reproductive and recovery rates in impacted areas (Pietersen et al. 2014a). There is also very limited information available on the abundance and distribution of all species that are all regarded as data deficient (IUCN 2015, Boakye et al. 2016).

Eight extant species are recognised (Gaudin et al. 2009), with four species distributed (Figure 1) in the Afro-tropics (Giant ground pangolin *Smutsia gigantea*, Temminck's ground pangolin *S. temminckii*, Black-bellied pangolin *Phataginus tetradactyla* and White-bellied pangolin *P. tricuspis*) and four species in the Indo-malayan regions of Asia (Indian pangolin *Manis*

crassicaudata, Philippine pangolin *M. culionensis*, Sunda pangolin *M. javanica* and Chinese pangolin *M. pentadactyla*). All species are considered to be threatened on the International Union for Conservation of Nature Red List (IUCN 2015), with the four African species listed as *Vulnerable* (Pietersen et al. 2014b, Waterman et al. 2014a, 2014b, 2014c) and the Asian species listed as *Endangered* (two species) (Baillie et al. 2014, Lagrada et al. 2014) and *Critically Endangered* (two species) (Challender et al. 2014a, 2014b). All species have now been up listed from Appendix II to Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) at 17th meeting of the Conference of the Parties (CoP17), which bans all international trade and provides for better domestic protection in Asia (Dixon and Weiskotten 2016). Therefore, effectively enforcing the existing legislation to address this unsustainable global criminal enterprise is needed. Reliable and accurate species identification is crucial for forensic investigation of cases where only processed animal parts are confiscated to assist with the monitoring and legal protection of pangolins.

Studies that applied DNA technologies for forensic wildlife species identification have shown that species and even populations can be distinguished with mitochondrial DNA (mtDNA) genes such as cytochrome c oxidase 1 (CO1), cytochrome *b* (Cyt *b*) and the control region (D-loop) (Hsieh et al. 2001, Branicki et al. 2003, Ogden and Linacre 2015). For example, the COI gene, which is also considered the standard DNA barcoding region for species identification (Hebert et al. 2003), has shown high levels of distinction among wildlife species (Dawnay et al. 2007, Mwale et al. 2015). While applications of DNA technologies in forensic crime investigation have been conducted for pangolin bush meat and scales (Hsieh et al. 2011, Gaubert et al. 2015, Zhang et al. 2015), these have mainly had a limited species representation, an Asian focus or have only employed analysis of a single mtDNA gene.

Furthermore, some published GenBank (National Center for Biotechnology Information) sequence data for pangolin species are incorrect and are based on taxonomically misidentified specimens making species assignments unreliable (Hassanin et al. 2015, Gaubert and Antunes 2015). Reference data for different gene markers that would enable reliable forensic identification of all African pangolin species is still lacking at present for legal enforcement of wildlife crimes involving pangolin poaching and seizures.

In this study we report on forensic species identification of a seizure of pangolin scales in Hong Kong using the COI barcoding genes, and Cyt *b* and D-loop gene regions. Our analysis includes (1) verification of the mtDNA test using Barcode of Wildlife Project (BWP) DNA barcoding reference samples to distinguish between pangolin species, (2) identification of species and the origin of confiscated samples, and (3) analysis of species composition of the confiscated samples.

Materials and Methods

Sampling and DNA extraction

Reference tissue voucher specimens (n=15) of three African pangolin species viz. *S. temminckii*, *P. tetradactyla* and *P. tricuspis*, were available from the National Zoological Gardens of South Africa (NZG) (see www.barcodewildlife.org) species reference database (Mwale et al. 2015) (Table 1). All voucher specimens were identified by a taxonomic expert (Ray Jansen; African Pangolin Working Group). Tissue samples of *P. tetradactyla* and *P. tricuspis* were collected in West Africa (Figure 2A) (Boakye et al. 2016), while *S. temminckii* samples were mainly collected in South Africa (Du Toit et al. 2014) (Table 1). In addition, reference samples were supplemented with sequences of *S. gigantea* and two Asian pangolin species (*M. javanica* and *M. pentadactyla*) retrieved from GenBank (Qin et al. 2012,

Hassanin et al. 2015) and the Barcode of Life Data (BOLD) Systems version 4 (beta) for the COI gene (Ratnasingham and Hebert 2007). These sequences were carefully selected to exclude samples where species misidentifications have been reported by recent publications (Hassanin et al. 2015, Gaubert and Antunes 2015). Suitable DNA sequences were available for six of the eight recognised pangolin taxa (Table 1) for testing species boundaries and identification of the unknown scales.

The unidentified pangolin scales are a subset of 3.3 tonnes of scales that were illegally traded and were confiscated by the CITES Management Authority in Hong Kong between 2014 and 2015, and are thought to have originated from Africa. A representative sub-sample of these confiscations consisting of 10 bags, each representing a different consignment with a scale net weight of 27.822 kg, was exported to the National Zoological Gardens of South Africa (NZG) for analysis. Each of the bags was accessioned and assigned a unique NZG biobank accession number. The contents of each bag (Figure 2B) were visually sorted into distinct scale types and were assigned to a species by the taxonomic expert based on their shape, colouration and morphology. A maximum of five samples per scale morph-type (putative species) were selected from each of the 10 bags for molecular characterisation.

Scale samples were pulverised using a dental electric micro-motor drill (Zhengzhou Xinghua Dental Equipment, Henan, China). DNA was extracted using the QIAamp DNA Investigator[®] Kit (Qiagen Inc., Valencia, California) following the manufacturer's instructions for degraded samples. DNA quantification for purity, concentration and yield was determined by using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Lithuania). Extracted DNA was stored at -20°C.

PCR amplification and sequencing

Polymerase chain reaction (PCR) was performed in 25 µl reactions that consisted of 5-20 ng of template DNA, 12.5 µL 2X DreamTaq PCR Mastermix (Life Technologies), 10 pmol of each primer (Table 2) and ddH₂O. The thermal cycling conditions were done according to published sources and optimisations as indicated in Table 2 (Kocher et al. 1989, Folmer et al. 1994a). PCR products were visualised on 2% agarose gel before being purified using the ExoSAP protocol (Thermo Scientific, Lithuania). Purified PCR products were cycle sequenced using the BigDye v3.1 Terminator Kit (Applied Biosystems, Foster City, CA) and then purified with a ZR DNA Sequencing Clean-up Kit (Zymo Research Corporation). Sequencing products were visualised with an ABI 3500 genetic analyser (Applied Biosystems, Foster City, CA).

Sequence assembly and phylogenetic analyses

Consensus sequences for each mtDNA gene were edited and assembled separately and then aligned using MUSCLE (Edgar 2004) in GENEIOUS v8.1.6 (Biomatters, LTD, Auckland, NZ). All mtDNA haplotypes were deposited in GenBank (Table 1). The best-fit model of nucleotide substitution for each dataset was estimated under the Bayesian Information Criterion (BIC) implemented using jMODELTEST 2.1.7 under default parameters (Guindon and Gascuel 2003, Darriba et al. 2012). Neighbour-joining (NJ) analyses were conducted using the selected models of evolution (with invariable sites) in GENEIOUS for all the gene and concatenated datasets. The topology of the NJ tree was confirmed with Bayesian Markov Chain Monte Carlo (MCMC) analyses (BI) as implemented in MRBAYES 3 (Ronquist et al. 2012) in GENEIOUS. Default settings were employed and simulations were run for 1,100,000 generations (Burn-in = 100,000) until the standard deviation of split frequencies was below 0.01.

Species delimitation in pangolins and forensic identification

Species distinction was evaluated by using the Basic Local Alignment Search Tool (BLAST) of the NCBI searchable database in GENEIOUS, and phylogenetic analyses of sequence characters. The NCBI BLAST (Altschul et al. 1990) uses an alignment program to determine the identity of unknown organisms based on pairwise DNA nucleotide comparisons (percentage matches) of gene sequences accessioned by researchers into this database. The phylogenetic analyses used two different approaches: (1) species tree analyses using each gene and annotated genome sequences and (2) analysis of a concatenated dataset (c-mtDNA) of all three gene fragments. The concatenated dataset was tested for congruence using the Incongruence Length Difference (ILD) Test in PAUP* v. 4.0b10 (Swofford 2002), where critical values for the test are between 0.01 and 0.001 (Farris et al. 1994, Cunningham 1997). Published mtDNA genome gene sequences (Table 1) from the three closely related orders suggested by Hassanin *et al.* (Hassanin et al. 2015) were used to root the trees.

Species boundaries were verified from the phylogenetic trees using the Species Delimitation Plugin (SDP) in GENEIOUS (Masters et al. 2011) and a standard barcoding gap analysis for the COI data based on Kimura 2-parameter (K2P) distances using the Automatic Barcode Gap Discovery (ABGD) method (Puillandre et al. 2012) with default parameters. The SDP delimits species by evaluating the phylogenetic exclusivity or monophyly of clades by testing the probability of this monophyly occurring by chance in a coalescent process (Masters et al. 2011). SDP also assesses the probability with which a putative species can be diagnosed successfully on a phylogenetic tree by comparing intra- and interspecific genetic distances among well-supported monophyletic clades (Bootstrap $\geq 70\%$).

The ABGD method uses several prior thresholds for the partitioning of sequences into primary species based on the “barcode gap” that compares the distribution of pairwise differences between intraspecific and interspecific diversity among different species (Hebert et al. 2003) without an *a priori* species hypothesis. The ABGD analysis estimates the relative gap width (X) and the minimum and maximum values of prior intraspecific divergence (P), which are used to detect the barcode gap using the default K2P model. Default settings of prior minimum genetic distances ranging from 0.001 to 0.1 on the ABGD website (<http://www.wabi.snv.jussieu.fr/public/abgd>) as these default *P*-values typically produce a range of OTU counts (Puillandre et al. 2012). In both delimitation methods, a species is distinct from its nearest neighbour (NN) if its maximum intraspecific distance is less than the distance to its NN sequence. Barcode gaps between well-supported clades of haplotypes identified by NJ and BI were taken as an indication of separate Molecular Operational Taxonomic Units (MOTUs). Therefore, barcode gaps and sequence divergences between well-supported clades identified by phylogenetic analyses were taken as an indication of separate species or OTUs.

Results

Species reference dataset and DNA barcoding analyses

Sequence alignment yielded 600, 400 and 576 base pair (bp) sequence fragments for COI, Cyt *b* and D-loop, respectively (Table 2). The HKY85 model (Hasegawa et al. 1985) was selected as the best-fit model of evolution for all gene fragments using jMODELTEST. The topologies of the NJ and BI gene trees and concatenated mtDNA dataset (Figure 3) were similar with regards to species relationships and clusters. All six reference species (*P. tricuspis*, *P. tetradactyla*, *S. gigantea*, *S. temminckii*, *M. javanica* and *M. pentadactyla*) were recovered and well-supported with high (>70%) bootstrap support (BS). Interspecific p-distance estimates between all species clades were high, ranging from 0.100–0.188 (COI) and

0.10–0.20 (Cyt *b*) (Tables 3 and 4) and 0.048–0.125 (D-loop). African specimens were also recovered as monophyletic (96–100% BS: all genes) sharing a most recent common ancestor (MRCA) that was distinct from the Asian pangolin lineage (*Manis* spp.). All four African pangolin species were further recovered as nearest neighbour taxa or sister-taxa within the two African genera (Figure 3). The concatenated reference dataset (Supplementary, Figure 1) of all genes (ILD tested) produced a fully resolved tree with strong bootstrap (>91%) and posterior probability (>0.99) support for monophyletic distinction among all species and genera, indicating that a combined gene analysis provides better phylogenetic signal.

The results of the SDP for COI, Cyt *b* and D-loop analyses using the phylogenetic trees (BI) were similar and are summarised in Table 4. The SDP analyses of all genes provided support for the distinction of all six species having high values for the two *P* (ID) estimates and their mean probabilities at 95% confidence intervals (Table 3). This result indicated that the species reference samples were significantly different ($P < 0.05$) from each other and represented distinct species under the same coalescent model of evolution. The mean intraspecific p-distances ranged from 0.001–0.055 (all mtDNA genes) among all species except for *M. javanica* and *P. tricuspis* (COI data: 0.037 to 0.030 respectively), which had higher maximum intraspecific divergences suggesting geographic sub-structuring. The observed trend of higher sequence divergence was also reflected in the Cyt *b* (Table 4) and D-loop (not indicated) analyses for *P. tricuspis*. The ABGD analyses was robust when comparing specimens of the same species and revealed six genetic groups (MOTUs) that corresponded with the six putative species (Figure 4) being distinct from 3.59–5.99 % COI divergence (Figure 4). The mean overall interspecific p-distance was significantly different ($P < 0.05$) and high (20.0–22.8%) between the three genera, providing support for delimitation at the genus level within Manidae.

Identification of unknown scales – Species assignment tests

DNA from 53 confiscated scales was extracted for species identification. The BLAST searches of all three genes indicated that the scale sequences all had 88–100% pairwise identity with six pangolin species (*P. tricuspis*, *P. tetradactyla*, *S. gigantea*, *S. temminckii*, *M. javanica* and *M. pentadactyla*) and unidentified Asian records (*Manis* sp.; 100% match; Table 5). However, examination of these low pairwise matches (88–90%) within species showed that these matches were all for comparisons with misidentified GenBank records incorrectly accessioned as *M. pentadactyla* and *P. tetradactyla*, that have been noted by recent studies (Hassanin et al. 2015, Gaubert and Antunes 2015). Although the COI match to *S. temminckii* was also low (Table 5: 89–90%), this match was considered to be an interspecific match within *Smutsia* based on the genetic diversities in the reference data (Table 3). Exclusion of low pairwise matches (interspecific) and misidentified samples improved the BLAST matches to a minimum of 93.4% (COI), 95.3% (Cyt *b*) and 93.2% (D-loop). The lowest NCBI BLAST matches (89.0–93.5%) were all among *Manis* and *Phataginus* species records probably due to high intraspecific divergence (COI) and lack of reference material for the D-loop. Furthermore, the high range variation within *P. tricuspis* (93.4–100 %) which has been noted in the literature (Hassanin et al. 2015) further suggests genetic sub-structuring. Matching sequence identity between scale sequences and the verified reference samples (this study) further improved the accuracy (95–100%) in matching scales to only four species, *P. tricuspis*, *P. tetradactyla*, *S. gigantea* and *M. javanica* (Table 4). There was no accurate match between the unknown scales and either *M. pentadactyla* or *S. temminckii* (Figure 5).

The combined SDP analyses based on reference and unknown scale phylogenetic analyses that tested the probability that the putative species clades had the observed degree of

distinctiveness, was still higher than the 0.05 threshold (0.5–0.12). The recovered six putative species clades (including scales) had Rodrigo's $P(\text{RD})$ values of <0.05 (Table 4, Figure 4) with Rosenberg's P_{AB} values being significant for all African species ($P < 0.05$), but not for the *M. javanica* species clade ($P > 0.11$), for all datasets. Rodrigo et al. (Rodrigo et al. 2008) define distinctive clades as those that have $P(\text{RD})$ values <0.05 . High values support species distinction while lower values may suggest cryptic speciation for branching events that would be expected under different coalescent modes (Masters et al. 2011). This result may therefore support the presence of other Asian species among these scales in the "*M. javanica*" clade (Figure 5).

All of the 10 pangolin bag samples were genetically identified as originating from both African and Asian pangolin species (Table 5). Six of the confiscated bags were of an African origin for three species (*S. gigantea*, *P. tetradactyla* and *P. tricuspis*) while four were of Asian origin, representing *M. javanica* (100% node support with reference sequence) and other Asian species (*Manis* spp.) whose identity could not be verified due to lack of verified species reference data. Species composition analyses indicated that some of the confiscated samples represented multiple pangolin species within one bag (Table 5). One bag (NZG 46070) had multiple *Manis* species from Asia while two of the bags contained the two African tree pangolin species (*P. tetradactyla* and *P. tricuspis*).

Discussion

The results of the phylogenetic analyses (NJ and BI) and independent methods with *a priori* grouping (ABGD and SPD) that were used to investigate species diversity were generally congruent among the gene fragments and had identical patterns with regards to species identifications. This consistent result for the reference database was critical to ensure accurate

and reliable assignment of samples of unknown species. This is especially important as the current GenBank database of published pangolin sequences has limited information on African species, with inaccurate information for several pangolin species (Hassanin et al. 2015, Gaubert and Antunes 2015) that can lead to poor confidence in species assignments. The observed interspecific divergence estimates of the reference database (minimum of 10% between species) was higher than the 2% that has been observed for reliable distinction among species of vertebrates for the COI and Cyt *b* regions (Hsieh et al. 2001, Hebert et al. 2003). These values were also within the range observed for D-loop sequences among pangolin species (Zhang et al. 2015). The level of sequence variation between individuals within *M. pentadactyla*, *M. javanica*, *S. gigantea* and *S. temminckii* was lower than that observed in *P. tricuspis* (up to 7% BLAST mismatch) suggesting genetic sub-structuring in this latter species. Potential cryptic speciation has been reported in this species (Hassanin et al. 2015), where these authors identified a unique lineage for a Gabon sample in their genomic analyses. Although high intraspecific variation was observed, accurate identification of species was still possible as the unique lineages in *P. tricuspis* were monophyletic and shared a MRCA. Furthermore, the intraspecific pairwise differences were much lower than the interspecific values and were within the expected limit of a “barcode gap” (Puillandre et al. 2012).

The observed differences in the phylogenetic relationships among pangolin species in the COI and Cyt *b* trees for *S. gigantea* and *S. temminckii* could be attributed to a single locus analysis that can be influenced by incomplete lineage sorting (Hebert et al. 2003), as analysis of the concatenated data produced a resolved tree at all taxonomic levels. This study therefore reinforces the utility of DNA barcoding for species identification using both COI and Cyt *b* gene regions. The successful use of mtDNA markers in forensic applications for the

identification of unknown animal samples to species, genus and family levels has been demonstrated by several studies (Hsieh et al. 2001, Verma and Singh 2002, Ogden and Linacre 2015). For example, Verma and Singh (2002), demonstrated the use of Cyt *b* in the identification of 221 species of animals for forensic cases of processed unknown animal parts. The advantage of mtDNA analysis is that the technology is cost effective and simple enough for individual laboratories even with poor resources to compare sequences for thousands of species (Ratnasingham and Hebert 2007).

Furthermore, results of this study indicate that forensic identification of all African pangolin species are reliable and effective using the COI, Cyt *b* and D-loop mtDNA gene regions. The gene trees of the combined reference and scale sequence dataset support the monophyly of all identified African groups that were in agreement with current taxonomy with very high support values (e.g. >94% for Cyt *b*). These monophyletic clades were also different from each other with high levels of divergence providing accurate species identification (>10% COI divergence between clades). The mtDNA sequences of scales identified as being from an African origin clustered in the strongly supported monophyletic clades (90–100% bootstrap support) of three species, viz. *S. gigantea*, *P. tetradactyla* and *P. tricuspis*, to the exclusion of sequences from Asian pangolin species. These three African species have overlapping distributions throughout most of their range in central and western Africa (IUCN 2015). The absence of *S. temminckii* in the confiscation and analyses may suggest that these specific African illegal trade consignments were very likely not of a southern or East African origin. *Smutsia temminckii* has the largest distribution range of the four African species and is the only pangolin species occurring in southern Africa and over most of East Africa. It is however important to note that this species' absence in the confiscation could also indicate a reduction in the abundance of *S. temminckii* in parts of its range where it marginally co-

occurs with *P. tricuspis*. *Smutsia gigantea*, a sister taxon of *S. temminckii*, has also been reported to be rare and was only identified in one bag, suggesting that species of this genus may occur at much low densities (Pietersen et al. 2014b) due to previous exploitation events or due to anthropogenic pressure. In southern Africa, the decline in abundance has been mainly associated with habitat loss and transformation (Pietersen et al. 2014a), generally due to agricultural intensification and anthropogenic activities such as electrocutions (electric fencing) as well as on-going poaching and illegal trade (traditional medicines).

This analysis also suggests that all pangolin species, including African species (*S. gigantea*, *P. tetradactyla* and *P. tricuspis*), are targeted regionally and as a group globally with no discrimination among species on the black market. Of the 10 batches that were analysed, three were mixed species bags (*P. tetradactyla*, *P. tricuspis* and Asian *Manis* spp.) with seven batches being unique to a single species. The two Africa origin mixed species bags contained both of the co-occurring tree pangolin species, with *P. tetradactyla* having a range that completely overlaps that of *P. tricuspis* mainly in central Africa. The analyses also indicated the presence of several Asian species in the consignment. Some scale samples were imbedded in a strongly supported monophyletic clade with the verified *M. javanica* reference sequence and had 100% blast matches with unidentified *Manis* species. However, this clade was not supported in the SDP, suggesting missing data. Reference samples are therefore required for all Asian pangolin species which have either no reference material (e.g. *M. culionensis* and *M. crassicaudata*) or lack specific accurate gene region (*M. pentadactyla*) datasets in GenBank. However, the absence of a match for *M. pentadactyla*, even with verified reference Cyt *b* sequences, suggests that this trade consignment may be from a specific area in Asia and can be traced to origin with further analyses. The presence of multiple Asian species in one bag also suggests indiscriminate poaching within Asia for pangolins.

Conclusion

These findings suggest that the reported illegal trade confiscation could be from several sources in Africa and Asia and is therefore part of an organised illegal trade enterprise throughout the distribution range of pangolins. Knowing the species targeted by poachers and the areas where poaching activity may be happening is needed to develop and support effective conservation plans by authorities. However, our analyses were not able to refer the confiscated scale samples to any specific populations due to the absence of geographically representative reference material. In addition, population-level markers such as single nucleotide polymorphisms (SNPs) would be required to accurately refer scales of unknown provenance to specific populations. Our analyses suggest that geographic identification would be possible for most pangolin species due to the high levels of intraspecific variation that indicates sub-structuring in African species (*P. tricuspis*). Further analysis with geographic sampling and a SNP database for African and Asian species will be a powerful tool and will facilitate a better understanding of the geographic origin of pangolin derivatives in trade, allow for better monitoring of this trade and effective law enforcement concerning illegal trade activities involving pangolins.

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Draft

1 **Table 1.** GenBank accession numbers and sampling localities of DNA sequence data that was used in the species reference dataset and as out
2 groups. Reference pangolin species sequences (bold font) are published and verified genome or gene sequences of each species.

Species	Locality	COI	Cyt b	D-loop
<i>Smutsia gigantea</i>	Cameroon*	KJ192837-8	KJ193382	unavailable
<i>Smutsia temminckii</i>	Tanzania†	KP306515	KP306515	KP306515
	South Africa (NZG reference)	KX012661	Submitted	Submitted
<i>Phataginus tetradactyla</i>	Cameroon*	KJ192841-2	unavailable	unavailable
	Ghana (NZG reference)	KX012676, KX012678-79, KX012684	Submitted	Submitted
<i>Phataginus tricuspis</i>	Gabon†	KP306514	KP306514	KP306514
	Cameroon, Ghana & Nigeria*	KJ192992, KJ192849	KJ193401-02, KJ193526	unavailable
	Ghana (NZG reference)	KX012680-85	Submitted	Submitted
<i>Manis javanica</i>	Thailand†	KP306516	KP306516	KP306516
	Malaysia‡	KF007331	unavailable	unavailable
<i>Manis pentadactyla</i>	China§	KC690306-7	unavailable	unavailable
	Thailand	unavailable	KP261032	unavailable
			KP261034	
<i>Canis latrans</i>	USA, Nebraska‡	NC_008093	NC_008093	NC_008093
<i>Ceratotherium simum</i>	-	NC_001808	NC_001808	NC_001808
<i>Rhinolophus monoceros</i>	-	NC_005433	NC_005433	NC_005433

3 Reference sequences were sourced from: the NZG reference database, *=Gaubert et al. (2015), †=Hassanin et al. (2015), ‡=GenBank sequence, §=Qin et al.
4 (2012) and ||=Gaubert and Antunes (2015).
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Table 2. PCR conditions and primer sequences used for sequencing three pangolin mitochondrial DNA gene fragments. Sequence information for the different gene fragments are also indicated.

Gene	PCR cycling conditions	Primers	Source	Fragment Length	BIC best-fit model	Variable sites (PS)	Informative sites (PI)
COI	40 cycles: 30 s at 94°C, 40 s at 45°C (x5) & 51°C (x35), 90 s at 72°C	dgLCO1490, dgHCO2198 Pan6AF, PAN6AR	Folmer et al. 1994 Du Toit et al. 2014	600 bp	HKY+I (I=0.631)	200 (33.0%)	147 (24.5%)
Cyt <i>b</i>	15 cycles: 30 s at 94°C, 50 s at 60°C (x5) & 55°C (x10), 60 s at 72°C	Cytb_Univ1, Cytb_Univ2	Kocher et al. 1989	400 bp	HKY+I (I=0.631)	133 (33.2%)	109 (27.2%)
D-loop	30cycles: 30 s at 94°C, 30 s at 50°C, 45 s at 72°C	Pan_15A_F, Pan_15A_R	Hsieh et al. 2011	576 bp	HKY+G (G=0.267)	131 (22.7%)	114 (19.8%)
All genes	-	-	-	1576 bp	HKY+G (G=0.170)	430 (27.3%)	217 (13.8%)

11 **Table 3.** Interspecific sequence divergence estimates (mean ± standard error) for the COI (above diagonal) and Cyt *b* (below diagonal) gene
12 regions of the species reference dataset.

Species	<i>S. gigantea</i>	<i>S. temminckii</i>	<i>P. tetradactyla</i>	<i>P. tricuspis</i>	<i>M. javanica</i>	<i>M. pentadactyla</i>
<i>Smutsia gigantea</i>		0.115±0.013	0.183±0.016	0.160±0.016	0.150±0.014	0.180±0.016
<i>Smutsia temminckii</i>	0.103±0.017		0.185±0.016	0.188±0.016	0.182±0.015	0.177±0.015
<i>Phataginus tetradactyla</i>	0.134±0.018	0.161±0.019		0.150±0.014	0.149±0.014	0.100±0.012
<i>Phataginus tricuspis</i>	0.144±0.019	0.145±0.019	0.151±0.019		0.102±0.012	0.165±0.015
<i>Manis javanica</i>	0.162±0.016	0.160±0.016	0.174±0.016	0.106±0.011		0.145±0.013
<i>Manis pentadactyla</i>	0.199±0.022	0.196±0.022	0.200±0.021	0.179±0.021	0.154±0.016	

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Table 4. Summary statistics of the Species Delimitation plugin analyses for the COI and Cyt *b* gene region datasets among six pangolin species. The Cyt *b* values are indicated in brackets when different from the COI estimates. Scales ID=species identifications of confiscated scales that were identified during this study; SGI=*Smutsia gigantea*; STE=*S. temminckii*; PTR=*Phataginus tricusps*; PTE=*P. tetradactyla*; MJA=*Manis javanica*; MPE=*M. pentadactyla*,

Species reference	N	Intra Distance	NN	Inter Distance - NN	Intra /Inter	P ID(Strict) COI	P ID(Strict) Cyt <i>b</i>	Av (MRCA-tips)	Rodrigo's P(Randomly Distinct)	Rosenberg's P _{AB}
1: SGI	2	0.003	2: STE	0.113 (0.113)	0.030	0.58 (0.43, 0.73)	0.59 (0.44, 0.74)	0.0001 (0)	0.05	0.03 (0.02)
2: STE	4	0.010 (0.003)	1: SGI		0.090	0.81 (0.67, 0.95)	0.91 (0.79, 1.00)	0.0081 (0.0035)	0.05	0.03 (0.02)
3: PTR	6	0.030 (0.055)	4: PTE (SGI)	0.162 (0.177)	0.190	0.91 (0.85, 0.98)	0.80 (0.69, 0.90)	0.0309 (0.0527)	0.12 (0.70)	<10 ⁻⁴
4: PTE	8	0.009 (0.004)	3: PTR (SGI)	(0.168)	0.060	0.92 (0.81, 1.00)	0.92 (0.80, 1.00)	0.0081 (0.0052)	0.05	<10 ⁻⁴
5: MJA	2	0.029 (0.001)	6: MPE	0.175 (0.147)	0.170	0.51 (0.36, 0.66)	0.79 (0.61, 0.96)	0.0182 (0.0008)	0.08 (0.05)	0.11 (0.02)
6: MPE	2	0.003 (0.004)	5: MJA		0.020	0.58 (0.43, 0.73)	0.77 (0.60, 0.95)	0.0011 (0.0036)	0.05 (N/A)	0.11 (0.02)
Scales ID										
1: SGI		0.006	2: STE	0.006	0.127		0.84 (0.69, 0.98)	0.0043	0.05	4.08E-03
2: STE		0.011	1: SGI	0.011	0.127		0.81 (0.67, 0.95)	0.0085	0.05	4.08E-03
3: PTR		0.032	4: PTE	0.132	0.250		0.91 (0.86, 0.97)	0.0284	0.05	9.1E-9
4: PTE		0.015	3: PTR	0.132	0.110		0.92 (0.83, 1.00)	0.0161	0.05	9.1E-9
5: MJA		0.037	6: MPE	0.154	0.240		0.47 (0.32, 0.62)	0.0184	0.05	0.11
6: MPE		0.002	5: MJA	0.154	0.010		0.58 (0.43, 0.73)	0.0010	0.05	0.11

Intra/Inter = Ratio of Intra (genetic differentiation among members of a putative species) to Inter (genetic differentiation between the members of a putative species and the members of the closest putative species); NN = nearest neighbour; P ID(Strict) = mean (95% confidence interval) probability of correctly identifying an unknown member of a given clade using the criterion that it must fall within, but not sister to, the species clade in a tree; and Rosenberg's P_{AB} = probability of reciprocal monophyly under a random coalescent model among taxa.

24 **Table 5.** Species identification results from the amplified genes and top similarity matches (BLAST) of confiscated scales batches (NZG
25 numbers) to the sequences from NCBI (in brackets). The species matches in bold font indicate the NCBI sequence matches of records noted as
26 species misidentifications from publications that were within the range of closely related pangolin species.

Bag No.	NZG Biobank number	ID & Number of scales analysed (n)	COI (% BLAST match)	Cyt <i>b</i> (%BLAST match)	D-loop (%BLAST match)	Verified species ID (SDP/ABDG)
1	46066	FC288A-FC288E (5)	<i>P. tricuspis</i> (94.9)	<i>P. tricuspis</i> (99.1 - 99.4)	<i>P. tricuspis</i> (96.5 - 98)	<i>P. tricuspis</i>
2	46067	FC289A-FC289D (4)	<i>M. javanica</i> (98.2)	<i>M. javanica</i> (96.4 - 97.2) <i>M. pentadactyla</i> (96.7-97)	<i>Manis sp.</i> (91-98.5)	<i>M. javanica</i> & <i>Manis</i> species
3	46071	FC290A-FC290D (4)	<i>S. temminckii</i> (89-90%)	<i>S. gigantea</i> (99.6 - 100)	unavailable	<i>S. gigantea</i>
4	46072*	FC291A-FC291F (6)	<i>P. tricuspis</i> (95.7)	<i>P. tricuspis</i> (98.4 - 100)	unavailable	<i>P. tricuspis</i>
		FC292A,C-FC292E (4) FC292B (1)	<i>P. tetradactyla</i> (88.4)	<i>P. tetradactyla</i> (100) <i>P. tricuspis</i> (99.6)	<i>P. tetradactyla</i> (94.0)	<i>P. tetradactyla</i> <i>P. tricuspis</i>
5	46063*	FC293A-FC293D (4)	<i>P. tricuspis</i> (94.2)	<i>P. tricuspis</i> (99.2)	<i>P. tricuspis</i> (96.7)	<i>P. tricuspis</i>
		FC294A1-FC294E1 (4)	<i>P. tetradactyla</i> (99.0-100)	<i>P. tetradactyla</i> (97.8 - 98.5)	<i>P. tetradactyla</i> (93.2-93.5)	<i>P. tetradactyla</i>
6	46070*	FC294A2-FC294E2 (5)	<i>M. javanica</i> (99.3-100)	<i>Manis sp.</i> (100) <i>M. javanica</i> (98.7 - 99.1)	unavailable	<i>M. javanica</i> & <i>Manis</i> species
		FC295A-FC295E (5)	unavailable	<i>Manis sp.</i> (99.6) <i>M. javanica</i> (95.4 - 99.3) <i>M. pentadactyla</i> (95.4)	<i>Manis sp.</i> (89-89.8)	<i>M. javanica</i> & <i>Manis</i> species
7	46065	FC296A-FC296C (3)	<i>M. javanica</i> (100)	<i>M. javanica</i> (96.7 - 99.1) <i>M. pentadactyla</i> (98)	<i>Manis sp.</i> (97.8)	<i>M. javanica</i> & <i>Manis</i> species
8	46064	FC297A-FC297B (2)	unavailable	<i>M. javanica</i> (97.2)	<i>Manis sp.</i> (89.5-93.1)	<i>Manis</i> species
9	46068	FC298A-FC298B (2)	<i>P. tricuspis</i> (93.7 - 95) <i>P. tetradactyla</i> (95)	<i>P. tricuspis</i> (99.1-100)	unavailable	<i>P. tricuspis</i>
10	46069	FC299A-FC299D (4)	<i>P. tricuspis</i> (93.4 - 95) <i>P. tetradactyla</i> (95)	<i>P. tricuspis</i> (97.3 – 99.4) <i>P. tetradactyla</i> (98.2)	<i>P. tricuspis</i> (97.2)	<i>P. tricuspis</i>

27 * Indicates bags with mixed species and scales that were sorted as different morph types before analyses.
28

FIGURE CAPTIONS

Figure 1. Global distribution of pangolin species according to the IUCN Red Data list of species (IUCN, 2015). The sampling sites where the reference material for three African pangolin species was collected (⊙) are also indicated. *Inset map is for the Philippine pangolin distribution.

Figure 2: Pangolins from the bush meat trade in West Africa (A) and scales from the Hong Kong confiscation (B).

Figure 3. Interspecific and intraspecific relationships among African (black bars) and Asian (grey bars) pangolin species used as reference material for the Cyt *b* (A) and COI (B) datasets. Values on the branches are bootstrap support values (below) and posterior probabilities (above; only posterior probabilities >0.95 are indicated) for all the phylogenetic analyses. Support values of the D-loop dataset received 100% bootstrap and 1.00 posterior probability support for all clades and all species and are therefore not indicated. Black nodes indicate sub-structuring in *P. tricuspis*.

Figure 4. Results of the Automatic Barcode Gap Discovery (ABDG) COI analyses of the mtDNA COI gene using Kimura-2-Parameter (K2P) distances. This analysis was for prior intraspecific divergences for the reference dataset of six putative species based on the distance at which a barcode gap occurs.

Figure 5. Phylogeny of the pangolin scales that were confiscated in Hong Kong in comparison to the reference species dataset (bold) based on Cyt *b* mitochondrial DNA sequences. Neighbour-Joining bootstrap values (below) and Bayesian Inference posterior probability values (above) are indicated on the branches. Missing support values (-) indicate lack of significant BS or PP support for the clade. Clades that were not identified among the confiscated scales are indicated by blue bars.

SUPPLEMENT

Figure S1: Interspecific and intraspecific relationships among African (black bar) and Asian (grey bar) pangolin species used as reference material for the combined COI, Cyt *b* and D-

62 loop datasets. Bootstrap support (below) and Bayesian posterior probability (above) value
63 estimates are indicated on the branches. Black nodes indicate sub-structuring in *P. tricuspis*.
64
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Global distribution of pangolin species according to the IUCN Red Data list of species (IUCN, 2015). The sampling sites where the reference material for three African pangolin species was collected (dotted circles) are also indicated. *Inset map is for the Philippine pangolin distribution.

Figure 1
201x140mm (300 x 300 DPI)

A

B

Interspecific and intraspecific relationships among African (black bars) and Asian (grey bars) pangolin species used as reference material for the Cyt *b* (A) and COI (B) datasets. Values on the branches are bootstrap support values (below) and posterior probabilities (above) for the Automatic Barcode Gap Discovery (ABGD) COI analyses and the Cyt *b* data. Analyses of D-loop received 100% bootstrap and posterior probability support for all clades and all species, and these values are therefore not indicated.

Figure 3

171x112mm (300 x 300 DPI)

Results of the Automatic Barcode Gap Discovery (ABDG) analyses of the mtDNA COI gene using Kimura-2-Parameter (K2P) distances. This analysis was for the prior intraspecific divergences for the reference dataset of six putative pangolin species based on the distance at which a barcode gap occurs.

Figure 4
159x114mm (150 x 150 DPI)

Phylogeny of the pangolin scales that were confiscated in Hong Kong in comparison to the reference species dataset (bold) based on Cyt *b* mitochondrial DNA sequences. Neighbour-Joining bootstrap values (below) and Bayesian Inference posterior probability values (above) are indicated on the branches. Missing support values (-) indicate lack of significant BS or PP support for the clade. Clades that were not identified among the confiscated scales are indicated by blue bars.

Figure 5
1190x1587mm (96 x 96 DPI)