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Original research article

Molecular tracing of confiscated pangolin scales for conservation and illegal trade monitoring in Southeast Asia

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ABSTRACT

Despite being protected by both international and national regulations, pangolins are threatened by illegal trade. Here we report mitochondrial DNA identification and haplotype richness estimation, using 239 pangolin scale samples from two confiscations in Hong Kong. We found a total of 13 genetically distinct cytochrome c oxidase I (COI) haplotypes in two confiscations (13 and ten haplotypes respectively, with ten shared haplotypes between confiscations). These haplotypes clustered in two distinct clades with one clade representing the Sunda pangolin (*Manis javanica*). The other clade did not match with any known Asian pangolin sequences, and likely represented a cryptic pangolin lineage in Asia. By fitting sample coverage and rarefaction/regression models to our sample data, we predicted that the total number of COI haplotypes in two confiscations were 14.86 and 11.06 respectively, suggesting that our sampling caught the majority of haplotypes and that we had adequately characterized each confiscation. We detected substantial sequence divergence among the seized scales, likely evidencing that the Sunda pangolins were harvested over wide geographical areas across Southeast Asia. Our study illustrates the value of applying DNA forensics for illegal wildlife trade monitoring.

1. Introduction

Illegal trade of wildlife is a major threat to biodiversity (Corlett, 2007; Nijman, 2009; Rosen and Smith, 2010). Overexploitation driven by wildlife trade has pushed many species to the edge of extinction, as in the well-known cases of tigers, rhinos and elephants. South-East Asia is among the world's "wildlife trade hotspots", where the trade is usually in high volume (Li et al., 2000; Nijman, 2009; Phelps and Webb, 2015). It is estimated that illegal wildlife trade is worth US$2.5 billion a year in East Asia and the Pacific (UNODC, 2013). Monitoring and regulating wildlife trade has become a critical conservation priority (Wasser et al., 2008; Oldfield, 2013; Welton et al., 2013; Chan et al., 2015; Challender et al., 2015).

Pangolins (family Manidae) are heavily poached for their meat and scales, to supply the illegal food and traditional medicine trade (Challender, 2011; Challender and Hywood, 2012; Zhou et al., 2014). There are eight species of living pangolins (Gaubert, 2011), and four pangolin species in the genus *Manis* are found in Asia (Fig. 1; Appendix 1 in

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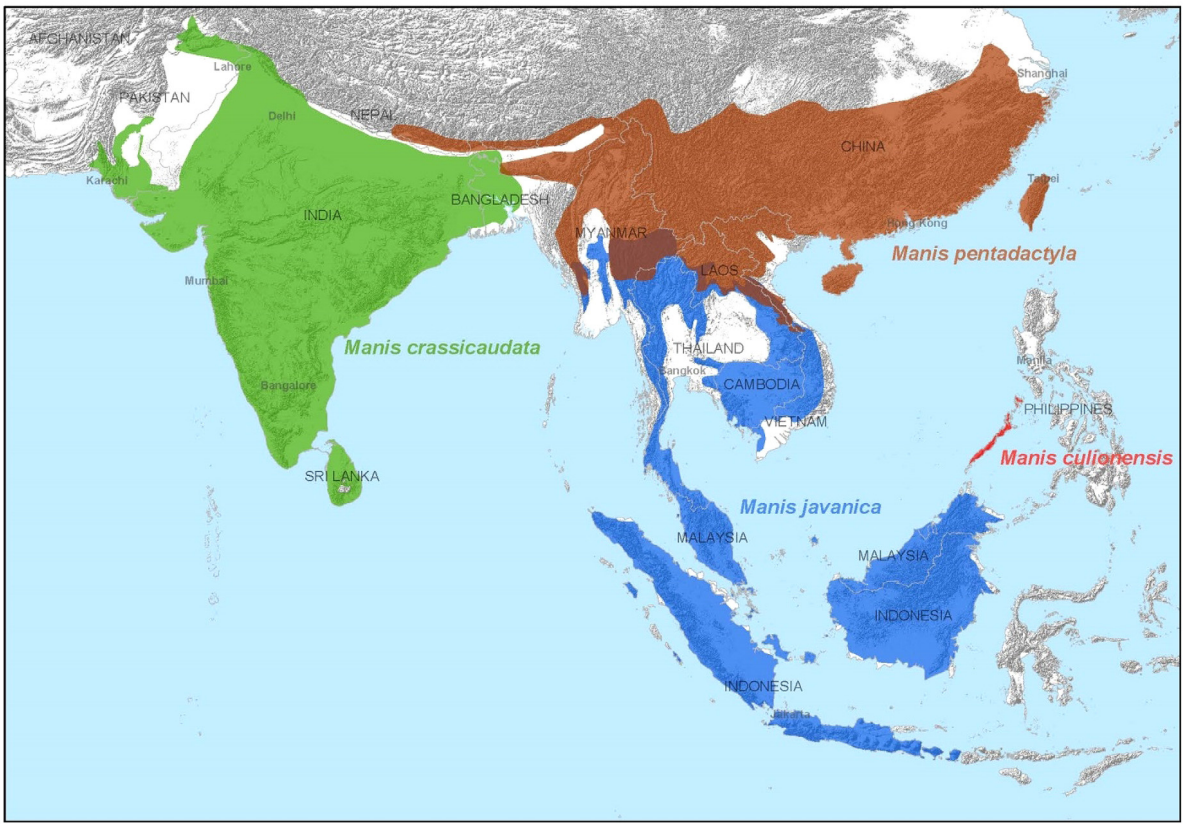

Fig. 1. Geographic distribution of the four Asian pangolins: the Chinese pangolin (*Manis pentadactyla*), the Sunda pangolin (*M. javanica*), the Palawan pangolin (*M. culionensis*), and the Indian pangolin (*M. crassicaudata*).

supplementary material): the Chinese pangolin (*Manis pentadactyla*), the Sunda pangolin (*M. javanica*), the Palawan pangolin (*M. culionensis*), and the Indian pangolin (*M. crassicaudata*). The remaining four pangolin species occur in Africa: the tree pangolin (*Phataginus tricuspis*), the long-tailed pangolin (*Uromanis tetradactyla*), the Cape or ground pangolin (*Smutsia temminckii*) and the giant pangolin (*Smutsia gigantea*). Pangolins are nocturnal animals that prey on ants and termites. They are "EDGE" (Evolutionarily Distinct and Globally Endangered; Isaac et al., 2007) mammals (constituting the Order Pholidota) that possess scale-covered bodies (Gaubert, 2011). Pangolins play an important role in controlling ant and termite populations in natural ecosystems (Swart et al., 1999; Wu et al., 2004). With their slow growth and low reproductive rates, pangolin populations are highly vulnerable to hunting. Many populations may now be locally extinct and recovery of the small number of individuals remaining in some severely impacted areas is likely to be slow (Wu et al., 2002; Newton et al., 2008).

Due to rapid declines in their populations, all pangolin species are listed as 'threatened' in the International Union for Conservation of Nature (IUCN) Red List (IUCN, 2015). They are also included in CITES Appendix II (CITES, 2013). Although pangolins are also protected by national and regional laws in their range states, they are still subject to illegal trade. Consumption of pangolins is driven by the folklore belief of their health benefits. However, pangolin scales consist of keratin, which has no proven pharmacological effects (Leader et al., 2008).

In the past decade, tons of pangolin scales and meat have been seized worldwide every year (TRAFFIC, 2013) and these seizure records are likely to represent only a small fraction of the massive illegal trade these species have been subject to (Pantel and Anak, 2010; Challender et al., 2015). In 2013, the illegal pangolin trade in Asia-Pacific was estimated to be valued at between US$100–150 million (UNODC, 2013). As the trade in pangolins raises serious legal and conservation concerns, accurate species identification is critical for trade regulation and law enforcement. However, it is difficult to confirm species identity from isolated scales (Challender, 2011).

Wildlife crime investigation has benefited from advances in DNA techniques which are increasingly used to assist cases (Ogden et al., 2009). Species or even populations can be distinguished by nucleotide substitutions in a selected fragment of DNA sequence such as the cytochrome c oxidase subunit 1 (COI) gene, the standard barcoding fragment for species identification (Hebert et al., 2003). Previous studies have shown application of DNA forensics in species identification from rhino horns (Hsieh et al., 2003), ivories (Ishida et al., 2013), whale and dolphin products (Baker et al., 1996), shark fins (Clarke et al., 2006), bushmeat including pangolins (Gaubert et al., 2015), and seized pangolin scales (Hsieh et al., 2011).

Here we report our investigation of two pangolin seizures in Hong Kong using the COI barcoding fragment. We chose the mitochondrial COI gene because it is used as the barcode to catalog all living species on Earth (Ratnasingham and Hebert, 2007), and it has shown great potential in wildlife forensics with ready-to-use universal primers (Dawnay et al., 2007; Eaton et al., 2010). Our analyses attempted to: (1) identify species and geographic origins of the seized pangolin scales; (2) estimate the total number of haplotypes in the confiscations to ensure that the confiscated scales were adequately characterized by our genetic analyses; (3) demonstrate the use of genetic information to assist in conservation and trade monitoring and (4) help to fill gaps in the global reference database for this mammalian group.

2. Material and methods

2.1. Taxon sampling

The study investigated two large seizures of pangolin scales that were made by the Customs and Excise Department of the Hong Kong Special Administrative Region (HKSAR) Government. Samples of scales were randomly removed from the consignments by the Agriculture, Fisheries and Conservation Department (AFCD) following a request for study samples from Kadoorie Farm & Botanic Garden (KFBG). The first confiscation in 2012 contained 224 kg of pangolin scales (Confiscation 1), whereas the second confiscation in 2013 included 312 kg of pangolin scales and 932 kg of carcasses shipped from Singapore (Confiscation 2). A total of 154 and 115 scale samples, weighing respectively 0.61 kg and 0.41 kg, were randomly taken from the two confiscations.

Scale samples were investigated for morphological characters, and inter-scale bristles were discovered in the samples, indicating that they belong to Asian pangolins (Heath, 1992). Our mammal experts were also able to distinguish scales which did not belong to Asian pangolins (based on physical features) from previous seizures in Hong Kong, and which turned out to belong to African species. Pangolin scales were occasionally connected by pieces of tissue, thereby presumably originating from the same individual animal. We separately weighed and labeled each scale-tissue clump. We estimated the total number of pangolins associated with each confiscation with the assumption that the average fresh weight of all scales on a *M. javanica* is 0.61 kg (Yang et al., 2010). The number of pangolin individuals in one confiscation was therefore calculated as: weight of confiscated scales (kg)/0.61 (kg).

Tissue samples were also taken from two de-scaled carcasses from Confiscation 2. The two carcasses were morphologically identified as *M. javanica* (Appendix 2 in supplementary material), and were further used as references for identification.

Reference DNA samples were also collected from five known provenance *M. pentadactyla* specimens originating from the wild in Hong Kong (Appendix 3 in supplementary material). Three *M. pentadactyla* individuals were rescued and delivered to the wildlife rescue center at KFBG as living specimens, whereas the other two specimens were dead carcasses found in Hong Kong's countryside. These five samples were valuable to the study because of their known provenance. Handling of pangolins and the collection of DNA samples was undertaken by rescue center staff under license from the authorities.

2.2. DNA extraction, amplification, and sequencing

We used DNeasy Blood and Tissue Kits (Qiagen) and followed the handbook protocol for DNA extraction of the samples. DNA concentration was determined using Nano Drop8000 (Thermo-scientific, USA), and then diluted to the working concentration of 10–20 ng/μl. We amplified the Cytochrome c oxidase subunit I (COI) gene from each sample using two separate primer sets to ensure accuracy. The first applied M13-tailed mammal cocktails from Ivanova et al. (2007). The second involved a new pair of primers (pangolin-COI-HZF3: 5′- AGATTTACAGTCTAATGCTT -3′ and pangolin-COI-HZR1: 5′- CCCATGTATCCAAAGGGCTCTT -3′) designed in Geneious Pro 6.1.6 software (Biomatters Ltd.). This primer set amplified 793 bp of the 5′ end of the COI gene and span the standard barcode region. The forward primer (pangolin-COI-HZF3) is located in tRNA(Tyr), which is a conserved region upstream of the COI gene. To compare with the reference data of *M. crassicaudata* (not represented by COI in Genbank), we amplified the first 402 bp of Cytochrome b (Cyt b) using the primer pair GVL14724—H15149 by Gaubert et al. (2011) in four scale samples (two samples from each of the COI haplotype H4 and H8).

DNA was amplified in the following PCR mixture for the new COI primers: 5 μl 5× Colorless GoTaq Flexi Reaction Buffer (Promega), 0.5 μl 10 mM dNTPs (Fermentas), 2.5 μl 25 mM $MgCl_2$, 1 μl 10 μM of each primer, 0.2 μl 0.02 U GoTaq Flexi DNA Polymerase (Promega), 1 μl DNA template, and ddH_2O. The thermal cycling profiles for amplifications were as follows: denaturation at 94 °C for 1 min, and 35 cycles of 94 °C for 30 s, 52 °C for 20 s, and 72 °C for 50 s followed by one cycle of 72 °C for 2 min. PCRs for COI mammal cocktails and Cyt b followed Ivanova et al. (2007) and Gaubert et al. (2011) respectively.

Amplification products were checked on 1.5% agarose gel and then sent to BGI Hong Kong for purification and sequencing on an ABI 3730xL Genetic Analyzer (Applied Biosystems).

2.3. DNA data analysis

The sample DNA sequences, together with the reference sequences of pangolins retrieved from GenBank (Appendix 4 and 5b in supplementary material) were assembled, edited and aligned in Geneious Pro 6.1.6 software (Biomatters Ltd.).

Sequence alignments were conducted with MUSCLE (Edgar, 2004) using default parameters, and subsequently checked by visual inspection. Final alignments have a sequence length of 600 bp in COI and 399 bp in Cyt b respectively. Outgroups were the domestic cat (*Felis catus*) for COI and the African tree pangolin (*Phataginus tricuspis*) for Cyt b.

We identified relationships among the sampled pangolin sequences and pangolin sequences obtained from GenBank in two different ways. First, Bayesian phylogenetic analysis was performed in MrBayes version 3.2 (Huelsenbeck and Ronquist, 2001) and maximum likelihood analysis in PHYML version 3 (Guindon and Gascuel, 2003) to infer phylogenetic relationships. The TRN + I model of evolution for COI and TPM2uf + G for Cyt b gene were selected based on the Akaike information criterion (AIC) in jModelTest 2.1.4 (Darriba et al., 2012). Second, to more clearly resolve relationships of the closely related *M. javanica* COI haplotypes, haplotype networks were constructed with TCS v.1.21 (Clement et al., 2000). The connection limit excluding homoplasic changes was set to 95% according to Hart and Sunday (2007).

Nucleotide divergences among *M. pentadactyla* and the seized scale clades of COI gene were quantified by MEGA 5 (Kumar et al., 2008) using between-groups mean p-distance and standard errors estimated by 500 bootstrap replications. Tests for genetic differentiation between the two confiscations were performed using the analysis of molecular variance (AMOVA) procedure in Arlequin 3.5 (Excoffier and Lischer, 2010).

2.4. Estimating the total number of pangolin individuals and COI haplotypes

We performed a series of analyses to determine the extent that the observed number of haplotypes in the sample of scales from each confiscation reflected the true haplotype abundance from the confiscations. To estimate the true number of COI haplotypes contained in each confiscation, we used both sample coverage and rarefaction techniques. Sample coverage methods (Huang and Weir, 2001) were adapted from techniques that have been used in ecological investigations to estimate the total number of species based on a single sample or a set of samples (Chao et al., 2000; Colwell et al., 2012). We estimated the total number of haplotypes (k) using the M1, M2, and M3 statistics described in Eqs. (3), (8), and (9), respectively, of Huang and Weir (2001).

The second approach involved the use of rarefaction (Kalinowski, 2004) to quantify the number of haplotypes that would be expected to be observed in samples of size $n = 1$ through $n = N - 1$, where N is the original number of individuals included in the sequence analyses. Rarefactions were based on Eqs. (1), (2a), and (2b) of Kalinowski (2004).

After completing the rarefaction stage, regression models were fit to the data to estimate the rate at which additional alleles would be observed in a data set as sample sizes increase. We fit five different models to the rarefaction curves including:

(1) a hyperbolic function of the form $y = (a * x)/(1 + (b * x))$,
(2) an exponential function of the form $y = a * (1 - b^x)$,
(3) a power function of the form $y = x^a$
(4) a logarithmic function of the form $y = 1 + a * \ln(x)$, and
(5) a simple linear function ($y = a * x + b$).

We calculated the residual sums of squared deviations (SSD_r) from each model and assumed that the model with the smallest SSD_r value would provide the best estimates of k (*sensu* Jackson et al., 2008). Note that the hyperbolic and exponential functions possess asymptotes at $y = a/b$ and $y = a$, respectively. We assume that the asymptotic value approximates the true value of k when either of these two models had the lowest SSD_r. The power, logarithmic, and linear functions have no asymptotes. Thus, if any of these models has the smallest SSD_r, extrapolation is required to infer a value of k based on the estimated model parameters assuming a population size of x. For the purposes of these analyses, we assumed underlying population sizes of 250, 500, 1000, and 2000 when generating any extrapolation-based estimates to encompass a range of possible values suggested by our estimates derived from scale weights (see Results).

The complete suite of analyses described above were performed separately for each confiscation, as well as for the sequences generated for both confiscations combined. All analyses described above were implemented using a computer program written in the Python programming language by MPM. Nonlinear regressions models were performed using the curve fitting routines of the "optimize.curve_fit" functions of the SciPy library for Python (www.scipy.org/scipylib; accessed 3 June 2014).

3. Results

3.1. Species identification

We obtained high quality COI sequences from 130 samples (out of 154 samples) in Confiscation 1 and 109 samples (out of 115 samples) in Confiscation 2. The weight for 130 samples in Confiscation 1 was 0.52 kg and 0.40 kg for 109 samples in Confiscation 2. A total of 13 haplotypes were identified in the 239 scale samples (Table 1).

The Bayesian tree and the Maximum Likelihood (ML) tree for COI possessed identical topologies. The COI phylogeny clearly separated the African from the Asian species with high statistical support (Fig. 2(a)). The clade of *M. pentadactyla* was well supported and five haplotypes were present in this clade. Four haplotypes were published

Table 1
COI haplotypes and number of samples with each haplotype in two confiscations.

		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13
Confiscation 1	Samples (#)	42	30	25	11	7	6	2	2	1	1	1	1	1
	Proportion (%)	31.34	23.10	19.23	8.46	5.38	4.62	1.54	1.54	0.77	0.77	0.77	0.77	0.77
Confiscation 2	Samples (#)	33	20	23	10	5	4	6	4	3	1	0	0	0
	Proportion (%)	30.28	18.35	21.10	9.17	4.59	3.67	5.50	3.67	2.75	0.92	0.00	0.00	0.00

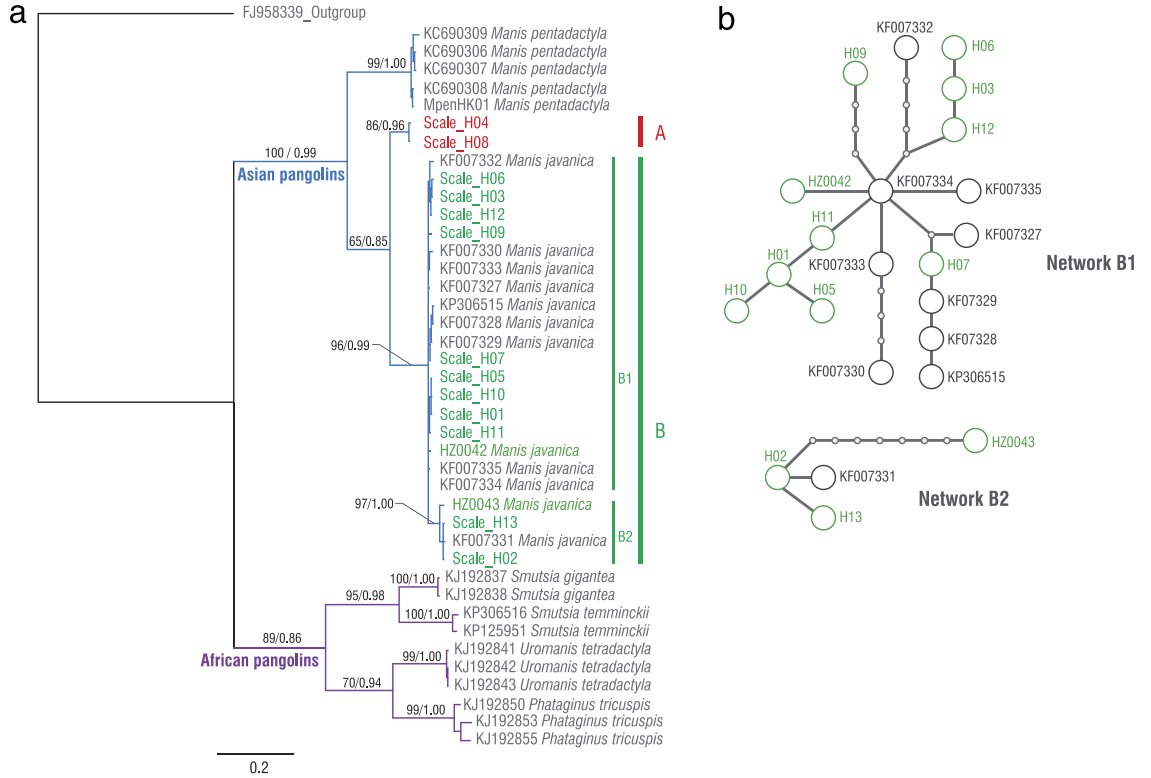

Fig. 2. (a) Bayesian tree showing the phylogenetic relationships among pangolin species and scale haplotypes using COI gene. Numbers above branches are values from the maximum likelihood tree/Bayesian posterior probabilities. Frequencies of scale haplotypes are listed in Table 1. Thirteen scale haplotypes are distributed in two clades (Clade A and B). Sequences from GenBank are listed as accession numbers. HZ0042 and HZ0043 are seized *M. javanica* carcasses. Outgroup taxon is *Felis catus*. (b) Haplotype networks for the COI gene of pangolin scale clades under the 95% parsimony criterion. The black dots correspond to the mutational steps between haplotypes obtained using the TCS program (Clement et al., 2000).

GenBank sequences of *M. pentadactyla* seized in mainland China, and our analysis identified an additional closely related haplotype in the Hong Kong specimens. None of these five COI haplotypes was recovered from the seized scale samples.

COI sequences from the confiscated scales and carcasses were divided into two distinct clades (Fig. 2(a)). Clade A consisted of two scale haplotypes (H4 and H8) which did not cluster with any reference data. From scale samples of H4 and H8, two samples of each COI haplotype were selected for Cyt b sequencing and two Cyt b haplotypes were found corresponding to H4 and H8. Phylogenetic analysis of Cyt b gene also suggested H4 and H8 were in a distinct clade, which was a sister clade to *M. javanica* and did not belong to *M. crassicaudata* and *M. pentadactyla* (Appendix 5a in supplementary material). Clade B corresponded to *M. javanica* and it consisted of 23 COI haplotypes. There was a sub-clade (four haplotypes) in Clade B divergent from the other 19 haplotypes. The mean p-distance between *M. pentadactyla* clade and the two scale clades (A and B) ranged from 12.6 to 13.2%, whereas it was 8.7% between A and B (Table 2).

3.2. Haplotype network analysis

Results from the statistical parsimony analysis were consistent with COI phylogenetic analyses, illustrating that Clade B had two separate haplotype networks (Fig. 2(b)). Network B1 included 19 haplotypes and Network B2 included four haplotypes.

Table 2
The between-groups p-distance calculated among *M. pentadactyla* and two COI scale clades showing the mean with the standard error.

	M. pentadactyla	Clade A
M. pentadactyla		
Clade A	0.126 (0.013)	
Clade B	0.132 (0.014)	0.087 (0.011)

Table 3
Estimated number of COI haplotypes from two confiscations of pangolin scales along with estimated derived from the combined data sets. Estimators M1, M2, and M3 are sample coverage estimators from Huang and Weir (2001). Regression-based models are described in more detail in the text.

Estimator	Confiscation		
	1	2	Combined
M1	13.52	10.09	13.16
M2	22.48	10.91	17.86
M3	31.87	11.06	20.62
Best-fit model	Hyperbolic	Hyperbolic	Logarithmic
Estimate type	Asymptote	Asymptote	Extrapolated
Estimated values	14.86	11.06	12.74 (extrapolation to 250) 14.21 (extrapolation to 500) 15.69 (extrapolation to 1000) 17.16 (extrapolation to 2000)

3.3. Comparison of two confiscations

In the 13 haplotypes identified from 239 scale samples, 10 haplotypes were shared between two confiscations (Table 1). The proportion of various haplotypes presented in each confiscation showed similar pattern, and the three most common haplotypes accounted for 73.67% and 69.73% of samples in Confiscation 1 and Confiscation 2 respectively. There were three haplotypes only found in Confiscation 1 and each of these haplotypes was represented by a single sample. Two unique haplotypes were found in two carcasses.

Based on frequencies of COI haplotypes, an analysis of molecular variance (AMOVA) indicated that all of the genetic variation occurred within confiscations, and there was no differentiation between the two confiscations (P value $= 0.677$ and Fst $= 0$).

3.4. Estimating the total number of pangolin individuals and COI haplotypes

The estimated number of pangolin individuals in Confiscation 1 and Confiscation 2 was 365 and 509 respectively based on the total weight of each confiscation and the average weight of scales of a *M. javanica* assumed to be 0.61 kg.

Hyperbolic function had the smallest SSD_r value and was identified as the best-fit model to the data of Confiscation 1 and Confiscation 2. Using the asymptote of the estimated curve, the regression analyses suggested that the total number of COI haplotypes in Confiscation 1 and Confiscation 2 were 14.86 and 11.06 respectively (Table 3). When sequences from both confiscations were combined, the logarithmic function was identified as the best-fit model and yielded extrapolated estimates from 14.21 to 15.69 for sample sizes of 500 and 1000. Sample coverage estimates tended to be more variable between estimators in Confiscation 1 than Confiscation 2. Estimates for Confiscation 2 ranged from 10.09 to 11.06, while Confiscation 1 generated estimates ranging from 13.52 to 31.87.

4. Discussion

4.1. Species identification

Our COI phylogeny illustrates the large genetic differentiation between African and Asian pangolins, and further shows that the confiscated scale samples are not from Africa. This result is consistent with the inter-scale bristles found in the confiscated scale samples, an attribute that is unique to Asian pangolins (Heath, 1992). Phylogenetic studies using mtDNA data (Gaubert and Antunes, 2015; Hassanin et al., 2015) and comprehensive suites of morphological characters (Gaudin and Wible, 1999; Gaudin et al., 2009) also suggest the division of African and Asian pangolins. Our COI tree clearly showed the separation between *M. pentadactyla* and *M. javanica*, in line with other phylogenetic analyses using mitochondrial markers (Gaubert and Antunes, 2015; Hassanin et al., 2015).

The majority of the confiscated scale samples belong to *M. javanica*, which is a widespread species (Fig. 1) and the most commonly traded pangolin in Asia (Challender, 2011; TRAFFIC, 2013). Although *M. pentadactyla* also has a long history of international trade, it was not detected in the two confiscations we studied. *M. pentadactyla* populations have continued

to decline in South China since the 1960s due to demand for medicine and food (Wu et al., 2002; TRAFFIC Southeast Asia, 2004), and that combined with habitat loss due to rapid development has resulted in local extinction of this species. Wild populations of *M. pentadactyla* still exist in Hong Kong, Taiwan, Nepal and the northern Indo-Burma region where additional research is required to better understand population ecology and genetic diversity of this dwindling species. Such information could be used to facilitate reintroduction of this keystone species to components of its historical range where it has been extirpated in the wild.

There are several possible identities for Clade A. One possible identity is *M. culionensis*, a sister-species of *M. javanica* (Gaubert and Antunes, 2005) for which no published genetic data is available. Alternatively, Clade A may correspond to an unknown lineage of *M. javanica*, or a cryptic pangolin species distributed within the range of *M. javanica*. The COI distance between Clade A and *M. javanica* was high (8.7%), possibly indicative of species-level divergence (Francis et al., 2010). Unfortunately, we could not obtain information on the geographic origin of those pangolins. A detailed population genetic study of *M. javanica* and *M. culionensis* will be needed to better trace the delineation of taxonomic lineages.

4.2. Provenance identification

Genetic data can be used to detect geographic origins of animal species if they have geographically structured genetic diversity (Ishida et al., 2013). However, we cannot pinpoint the origins of the seized scales due to limited reference data (see above) and unknown geographic distributions of genetic lineages in *M. javanica*. Also trade routing can invariably be complicated as has been seen in other illegally traded species such as Chelonians (Ades et al., 2000), making determination of origin point for many individuals and scale consignments almost impossible.

Most of the scale haplotypes can be found in both confiscations, suggesting they are collected from similar areas, and possibly originating from the same smuggling ring. Although our sample sizes for *M. pentadactyla* originating from Hong Kong were small, they carried a distinct COI haplotype. If additional analyses based on larger sample sizes corroborate this finding, then our data increases the potential for predicting the original geographic locations of illegal *M. pentadactyla* harvests. If each scale haplotype found in this study is collected from a particular geographic region, then the confiscated pangolins are highly likely to be harvested from wide geographical areas of Southeast Asia, and then gathered in central collection points for processing and shipping. In some cases, the illegal wildlife trade involves opportunistic, loosely connected networks of individuals (Warchol, 2004). However, given the large size of the two Hong Kong seizures, it is possible that the operation of the harvest and smuggling involve an intricate network of hunters, traders and criminal syndicates.

Discovery of multiple clades and large genetic divergence were also found from seized pangolin scales by using mitochondrial D-loop in Taiwan (Hsieh et al., 2011), and most of those scales are likely to belong to *M. javanica* (Gaubert and Antunes, 2015). As little is known about intra-specific genetic diversity of *M. javanica*, the present study has offered new insights into the cryptic genetic diversity of this species and suggests it is composed of different genetic lineages that may constitute different management units or species.

4.3. Estimating the number of pangolins and COI haplotypes

The total number of COI haplotypes predicted for the two confiscations are close to the number of COI haplotypes acquired from our samples. This result suggests that our sampling has caught most of the COI haplotypes and that we have adequately characterized the confiscations.

Estimating the number of pangolins from scale weight is difficult without accurate species identification because different pangolin species have variable body sizes and scale weights. Limited information is available for the weight of scales of a pangolin. Heath (1992) suggests the proportion of scale weight to total mass is around 25% in *M. pentadactyla*. Hong Kong Customs & Excise Department uses 0.45–0.55 kg/individual as an assumption to estimate the total number of pangolin individuals from confiscated scales (TRAFFIC, 2013). Yang et al. (2010) studied the smuggled *M. javanica* in China and found the average fresh weight of scales for a *M. javanica* is 0.61 kg. Among the scale samples from the two Hong Kong confiscations, there are also skin tissue, tail parts and claws attached to scales. We may have over-estimated the number of pangolin individuals in our confiscations by using 0.61 kg, which is for the weight of the scales alone.

4.4. Illegal trade and conservation of pangolins

The illegal wildlife trade has been catastrophic for pangolins and the ecosystems that they inhabit. Overharvesting of pangolins not only threatens their existence, but also affects the food web and local ecosystems because pangolins strongly influence the abundance and diversity of important invertebrates in these ecosystems (Swart et al., 1999; Sileshi et al., 2009).

The illegal trade of pangolin scales is largely driven by high demand and high profit. There is a high demand for pangolin scales for traditional medicines in Asia, and it was estimated that demand in China alone amounted to 200,000 pangolins per year (Wu et al., 2002). The price of pangolin scales on the black market has risen from HK$2000 to HK$5000 per kg over the past five years (Lo, 2014). However, it is becoming more difficult to find wild pangolins in Asia and although the

transportation distance is much greater, it has now become commercially viable for traders to exploit African pangolins through recently established smuggling networks in Africa (Challender and Hywood, 2012).

Monitoring and regulating wildlife trade is essential (Nijman, 2009). Our study demonstrates that DNA forensics is a powerful tool that can facilitate wildlife crime investigations (Alacs et al., 2009). Specifically, if regional authorities collect and maintain samples from confiscations for genetic investigations, then barcode genes such as Cyt b and COI may prove useful to investigations, especially given that there are validated primers and that much data now exists in GenBank available for comparison purposes (Gaubert and Antunes, 2015). The information may help to strengthen the monitoring and enforcement networks along the supply chain and assist in combating the illegal wildlife trade.

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Appendix A. Supplementary material

The following is the Supplementary material related to this article.

Appendix 1 shows photos of the four Asian pangolins.

Appendix 2 contains a comparative table of species-diagnostic characters among Asian pangolins, and photos of the two seized carcasses.

Appendix 3 contains a table of reference pangolin specimens from this study.

Appendix 4 shows detailed information on the GenBank sequences and scale haplotypes used in the COI phylogenetic tree.

Appendix 5 shows the Cyt b phylogenetic tree among Asian pangolin species, and information on the GenBank sequences and scale haplotypes used in the Cyt b tree.

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