



## Original Research Article

## Genetic identification of African pangolins and their origin in illegal trade

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## ABSTRACT

To track the illegal pangolin trade from Africa to Asia, we analyzed 1800 DNA samples from 30 seizures of African pangolin scales in Hong Kong during the period 2012–2016. We concluded that all four African pangolin species were present in trade, and that the white-bellied pangolin (*Phataginus tricuspis*) appeared most frequently (88.5%) in our samples. All six previously described phylogeographic lineages originating from the entire distribution range of *P. tricuspis* were found in the seizures, and the western central African lineage alone accounted for 67.1% of the samples of this species. Confirmed by modelling data, high DNA haplotype richness was present in most of the pangolin scale seizures, including those contained in small air parcels and large-volume sea shipments. Results suggest that African pangolins were hunted across large areas of their natural range and then delivered to a small number of trade transit hubs. Our study illustrates the utility of genetic analysis for characterizing the illegal pangolin trade and identifying the geographic origin of poaching hotspots.

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## 1. Introduction

The illegal wildlife trade is a multibillion-dollar business that has been recognized as a major threat to biodiversity conservation (Rosen and Smith, 2010). Each year, hundreds of thousands of plants and animal species are collected or hunted from the wild and traded across the world (Oldfield, 2003). High consumer demand, increasing prices, and low penalties are driving traders and hunters to search every corner of the globe for the last remaining populations of valued species (Challender et al., 2015).

Pangolins (Family: Manidae) are “EDGE” (Evolutionarily Distinct and Globally Endangered) mammals with important ecological roles (Isaac et al., 2007), and all pangolin species are listed in Appendix I of CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora). They are among the most heavily poached animals partly due to growing demand from East Asia for their scales for use in traditional medicine and meat for consumption (Challender, 2011; Heinrich et al., 2016; Shepherd et al., 2017). The four Asian species are facing high extinction risk due to decades of over-collection (Challender, 2011), but comparatively less is known about the four pangolin species endemic to Africa (Fig. 1). The African pangolins were primarily hunted for local consumption (Soewu and Sodeinde, 2015; Ingram et al., 2018), but

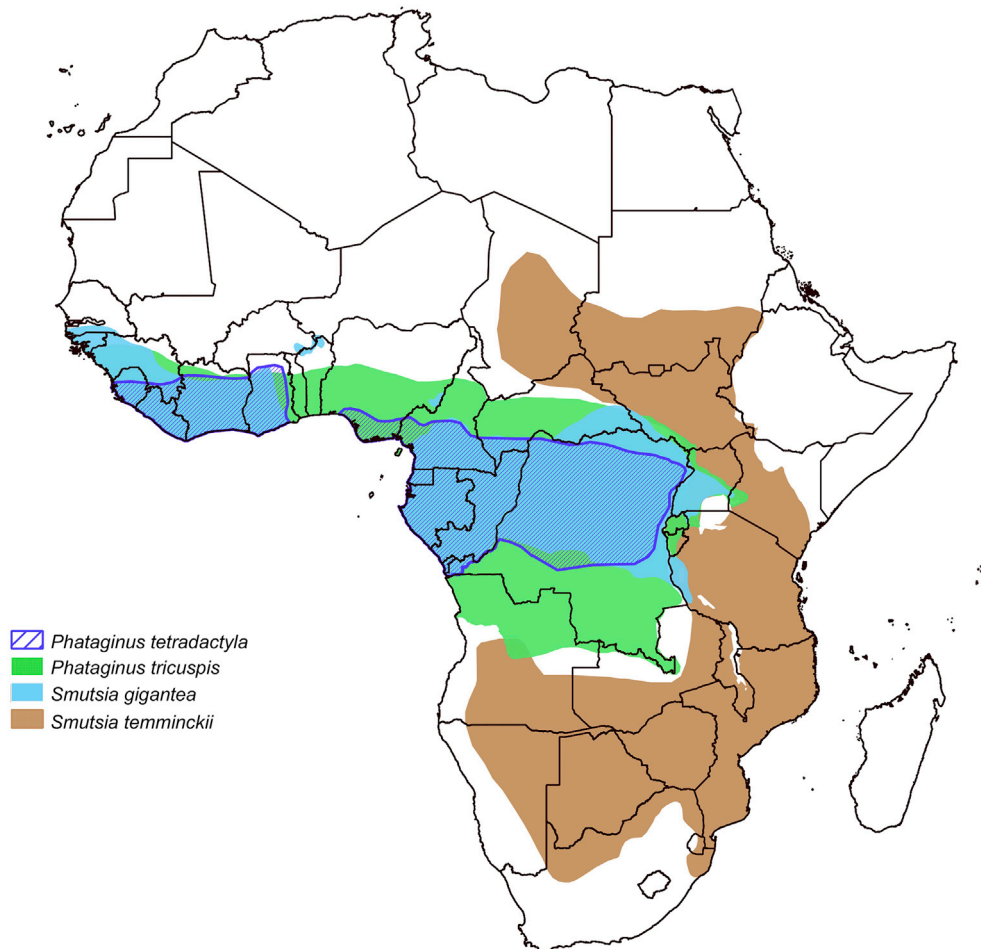
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there is an increasing intercontinental trade flow to Asia indicated by the huge quantities of scales seized during the last decade (Challender and Hywood, 2012; Heinrich et al., 2017; Ingram et al., 2019).

Authorities in Africa and Asia have taken measures to combat this illegal trade through anti-poaching actions, seizures, and prosecutions (Cheng et al., 2017; Ingram et al., 2019; Shepherd et al., 2017). Much effort has also been directed at understanding the trade and informing conservation measures. Catch data and market survey in Africa have been used to estimate the extent of exploitation, but the relationship between local hunting and international drivers is not well understood (Ingram et al., 2018, 2019). Seizure data analysis has revealed some of the trade routes to consumer markets, but little information is available about the species in trade and their geographic origins (Cheng et al., 2017; Heinrich et al., 2017). To identify conservation priorities, an underutilized but powerful approach would be to examine seizures on a large scale using DNA techniques to identify key species in trade and elucidate trade routes, that would then support law enforcement in key origin, transit and destination countries (Ogden et al., 2009; Wasser et al., 2015). DNA analysis has been successfully employed to help identify seized pangolin scales and to reveal traded species and genetic diversity in seizures (Mwale et al., 2017; Nash et al., 2018; Zhang et al., 2015). Origin identification is more challenging, but Gaubert et al. (2016) indicated that mitochondrial DNA markers had potential to identify source regions of the white-bellied pangolin (*Phataginus tricuspis*). Furthermore, when DNA techniques are combined with appropriate sampling design and analysis tools, it would be possible to characterize the trade and reveal underlying trade patterns.

In the present study, we analyzed African pangolin scales seized in the Hong Kong Special Administrative Region of the People's Republic of China (from now on referred to as Hong Kong), one of the most important transit hubs globally for African pangolins (Heinrich et al., 2017). DNA analyses and statistical modelling were employed to: (1) identify the key species in trade and the poaching hotspots, for the illegal pangolin trade from Africa to Asia; (2) estimate the number of species, phylogeographic lineages and DNA haplotypes using statistical modelling in order to determine if our sampling was adequate to characterize individual seizures; (3) quantify whether DNA haplotype richness increases with seizure volume.



**Fig. 1.** Range-wide distribution of the four African pangolins in sub-Saharan Africa according to the International Union for the Conservation of Nature Red List (IUCN 2020). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

## 2. Material and methods

### 2.1. Biological material sampled

We studied 30 consignments of pangolin scales originating from Africa that were seized by the Hong Kong Customs and Excise Department (C&ED) between 2012 and 2016. Each seizure had detailed record information for the export country, export mode (by air or by sea), seized volume and date (Table S1). One to 2 kg of samples, comprising up to a few thousand scales, were randomly removed from each seizure by officers of the Agriculture, Fisheries and Conservation Department (AFCD). If a consignment contained multiple packages, samples were randomly selected from different packages and pooled together. Sixty scale samples were then randomly taken from each seizure for DNA analysis.

### 2.2. DNA extraction, amplification, and sequencing

Subsampled scales were processed in accordance with established contamination control precautions and workflows. Negative controls were included in sample preparation, DNA extraction and PCR to monitor for possible contamination. Genomic DNA was extracted using DNeasy Blood and Tissue Kits (Qiagen) under a laminar flow cabinet following the manufacturer's protocol.

The mitochondrial cytochrome *b* (cytb) gene was selected to study the seized scales because it showed high accuracy for species and origin identification of African pangolins (Gaubert et al., 2015, 2016). Both 'universal' and pangolin-specific primer sets were used to amplify the same region of cytb gene for quality control. The 'universal' primer pair was GVL14724 - H15149 from Gaubert et al. (2015), and the pangolin-specific primer pair involved H15149 and a new forward primer (P\_L14707: 5'CTCTAACCATGACCTATGA) designed based on published mitochondrial genome data for pangolins in GenBank. PCR amplification for two primer sets followed Gaubert et al. (2015). Amplification products were checked on 1.5% agarose gel and then sent to BGI-Hong Kong for purification and bidirectional sequencing.

### 2.3. Genetic identification and sequence analyses

Successful sequences from seized samples were checked and edited in Geneious software (Biomatters Ltd.). The DNA haplotypes were defined based on substitutions within the first 402 bp of cytb gene with positions in the mitochondrial genome (14,127–14528), and then identified using FaBox 1.41 (Villesen, 2007). All haplotypes obtained from scale samples were submitted to GenBank (MN442125–MN442317) with associated laboratory reference identification codes (Table S2).

To construct a reference database for genetic identification, published cytb and mitochondrial genome data for four African pangolin species (*Phataginus tricuspis*, *P. tetradactyla*, *Smutsia gigantea* and *S. temminckii*) were downloaded from GenBank (Table S3). Species identification for scale samples was conducted using BLAST searches and phylogeny tree based identification.

Gaubert et al. (2016) indicated that there was phylogeographic structure associated with haplotype variation in *P. tricuspis*. Thus, to infer geographic origin of *P. tricuspis* samples identified in the seizures, haplotypes from seized samples and those from GenBank were combined for phylogeographic analysis. The neighbor-joining (NJ), maximum-likelihood (ML) and Bayesian trees were built in Geneious software (Biomatters Ltd.). The JC model was selected as the best-fitting evolutionary model for ML and Bayesian trees based on the Akaike Information Criterion (AIC) test implemented in jModelTest v. 2.1.6 (Darriba et al., 2012).

The relationships between all haplotypes of seized and reference samples for *P. tricuspis* were further assessed using the median-joining algorithm in NETWORK 4.6.1.1 ([www.fluxus-engineering.com](http://www.fluxus-engineering.com)). Based on phylogenetic and haplotype analyses, phylogeographic lineages (western Africa, Ghana, the Dahomey Gap, western central Africa, Gabon and central Africa) described by Gaubert et al. (2016) were assigned to the haplotypes of seized samples (Table S2).

### 2.4. Statistical modelling and analyses

To infer the number of species, phylogeographic lineages and haplotypes in each seizure, we used two approaches following Zhang et al. (2015). These analyses allowed us to determine if our sampling was adequate to characterize each seizure, and also if some species/lineages/haplotypes were not being detected. The first approach involved use of the M1, M2, and M3 statistics based on sample coverage methods outlined by Huang and Weir (2001). In the second approach, we performed rarefaction analyses (Kalinowski, 2004) to account for different sample sizes, and then fitted five regression models (hyperbolic, exponential, power, logarithmic, and simple linear function) to the rarefaction curves as per Zhang et al. (2015). After these analyses, models with the lowest value of the residual sums of squared deviations ( $SSD_r$ ) were selected and assumed to represent the best estimates (Table S4). The hyperbolic and exponential functions had asymptotes and we assumed that the asymptotic value approximated the true value when either of these two models had the lowest  $SSD_r$ . For the other three functions, the estimated number of haplotypes will be a function of the population size, where the complete seizure is in this case considered to be the population of interest. When one of the three non-asymptotic functions provided the best fit, size for each seizure was derived from scale weights as per Zhang et al. (2015), where 0.5 kg was considered to represent the average weight of scales removed from a seized pangolin individual (Cheng et al., 2017).

Paired t-tests were used to evaluate whether there were differences between the observed and predicted by modelling (M1, M2, M3 and the best model from regression-based approach) for species richness, the number of phylogeographic lineages and haplotype richness. Further, Spearman rank correlations were calculated to assess the relationships between the volume of seized scales (natural log transformed) with the number of haplotypes, derived from both observed and estimated data. Finally, t-tests were used to examine whether the number of haplotypes differed in relation to the mode of export (by air and by sea). These analyses were conducted in R version 3.4.2 (R Core Team, 2016).

### 3. Results

Good quality DNA sequence data was obtained from 1648 out of 1800 studied samples. All four extant African pangolin species were represented in the seized scale samples (Fig. 2A). *P. tricuspis* was represented by 1459 samples detected among the 30 consignment seizures, and accounting for the majority (88.5%) of the samples analyzed. The other three species were present in a relatively smaller number of samples/seizures: *P. tetradactyla* (119/19), *S. gigantea* (61/6), and *S. temminckii* (9/1).

Most (73.3%) seizures contained multiple pangolin species (Fig. 2A). Eighteen seizures (60%) had two species, with *P. tricuspis* and *P. tetradactyla* found together in 16 (53.3%) seizures. Four seizures (13.3%) had three species and eight seizures (26.7%) had one species detected.

A total of 169 cytb haplotypes were identified in scale samples of *P. tricuspis* (Table S2), and they were analyzed together with 38 reference haplotypes of 103 specimens from GenBank (Table S3). The three phylogenetic trees (NJ, Bayesian and ML) possessed similar topologies and all scale haplotypes were grouped into six phylogeographic lineages delineated by reference haplotypes (Fig. 3). NETWORK analyses also produced six haplotype networks which corresponded to the six phylogeographic lineages (Fig. 4).

The western central Africa lineage of *P. tricuspis* had 979 samples and 91 haplotypes found in 25 seizures, accounting for the majority of the samples for this species (67.1% and 53.8% of the samples and haplotypes, respectively) (Figs. 2B and 3). The other five lineages had a smaller number of samples/haplotypes/seizures. They were the Dahomey Gap (280/22/9), Gabon (111/34/13), Western Africa (73/15/2), Ghana (12/4/2), and Central Africa lineage (4/3/1).

Most (66.7%) seizures contained scales consisting of multiple phylogeographic lineages of *P. tricuspis* (Fig. 2B). Eighteen seizures (60%) had two lineages, with western central Africa and Gabon found together in nine (30%) seizures. Two seizures had three lineages detected together, and they were Western central Africa, Gabon, and the Dahomey Gap.

The remaining three species were represented by a smaller number of haplotypes as follows (Table S2): *P. tetradactyla* (16), *S. gigantea* (6) and *S. temminckii* (2). The reference samples of these three African pangolin species were geographically limited and not sufficient for origin identification.

The 30 seizures involved a total of 22,795.2 kg of pangolin scales, equivalent to 45,590 individuals based on the assumption that 0.5 kg of scales represents one seized pangolin (Cheng et al., 2017). These seizures would only have represented a part of the illegal trade for the study period due to inherent detection and reporting biases associated with seizure data (Underwood et al., 2013). The consignments were smuggled into Hong Kong from six countries by air or by sea (Table S1 and Fig. 2). Nigeria was the major export country involved in 20 seizures, followed by Cameroon (six seizures). Ivory Coast, Uganda and Ghana each were involved in one and Kenya in two seizures. 2013 saw the largest number of seizures (16 seizures), and all consignments arrived in Hong Kong by air. Sea shipments were more frequently discovered between 2014 and 2016. Although seizures of sea shipments were less frequent than seizures of air consignments (7 versus 23), they involved a significantly larger quantity of scales (21,589.2 kg) accounting for 94.7% of the seized scales used for this study (Table S1).

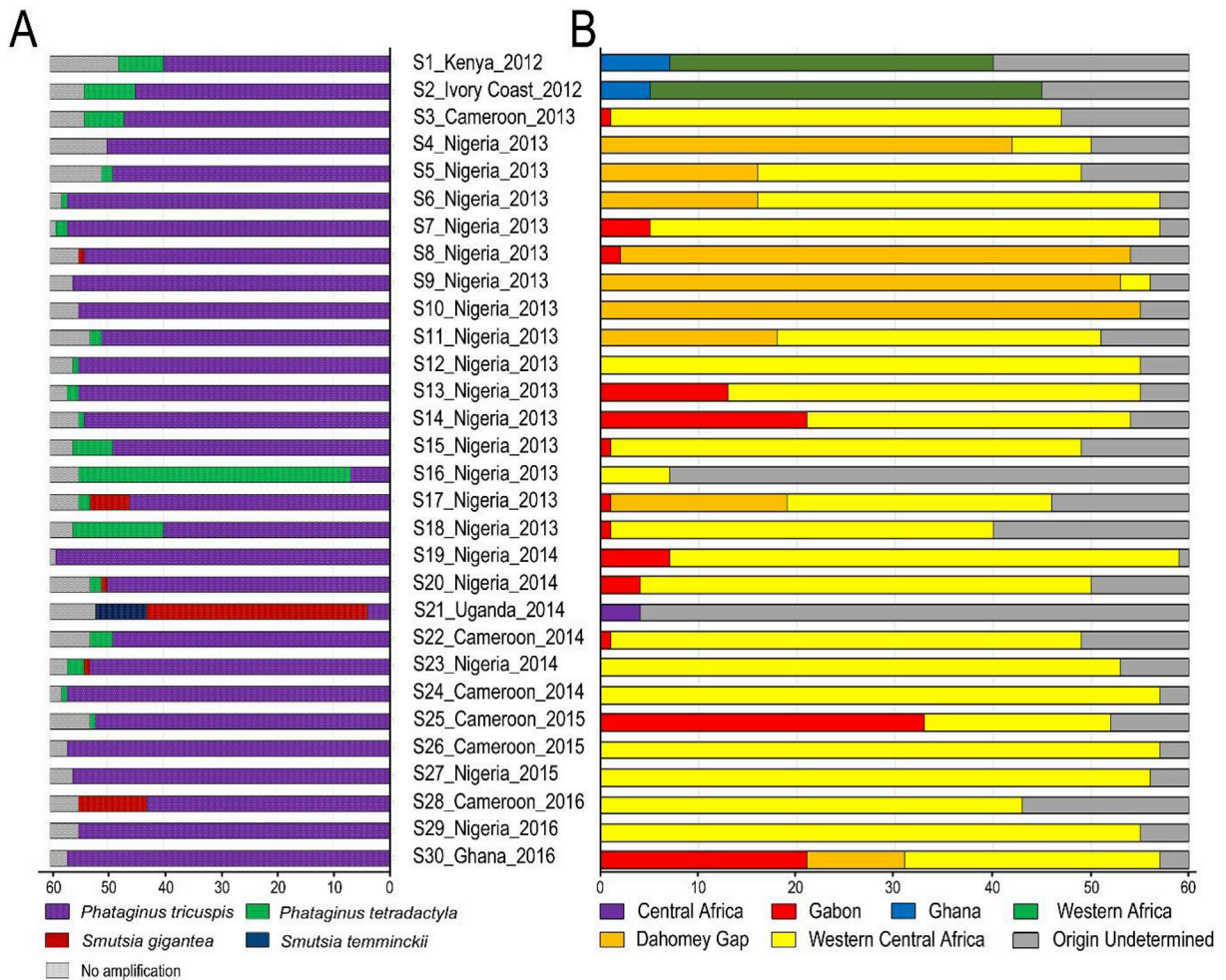
Estimates of the number of species, lineages and haplotypes in each seizure varied depending on the specific estimator used (M1, M2, M3 or regression-based approach; Table 1 and Table S4). Of the estimators employed, only the regression-based analyses showed no significant difference between observed and modelled values, although the regression-based estimates were very large in a few large-volume seizures (Table S4). By contrast, the M1, M2, and M3 estimates for the observed number of haplotypes were all significantly larger than the observed values, and the M3 estimates for number of species was also significantly larger than the observed value (Table 1). The seizure volume was not correlated with observed and the M1, M2, and M3 estimates, but correlated with regression-based best model for haplotype richness (Table 2). There was no difference in haplotype richness between scales exported by sea and by air for observed ( $p = 0.35$ ), M1 ( $p = 0.28$ ), M2 ( $p = 0.35$ ), M3 ( $p = 0.56$ ) and regression-based estimates ( $p = 0.30$ ). Instead, high haplotype richness was found in most seizures.

### 4. Discussion

The DNA analysis of the scales from 30 pangolin seizures in Hong Kong provides valuable information and insight regarding the illegal pangolin trade from Africa to Asia. The seizures of pangolin scales in Hong Kong represent a substantial percentage of the recorded global seizures (Heinrich et al., 2017), and our study includes samples from both large- and small-volume seizures.

The four African pangolin species are present in the global CITES database (Heinrich et al., 2016), and three species were detected in recent Hong Kong seizures by Mwale et al. (2017). Our detection of all four African pangolins in the seizures further suggests that Hong Kong is a key transit hub for the illegal pangolin trade from Africa to Asia (Heinrich et al., 2017).



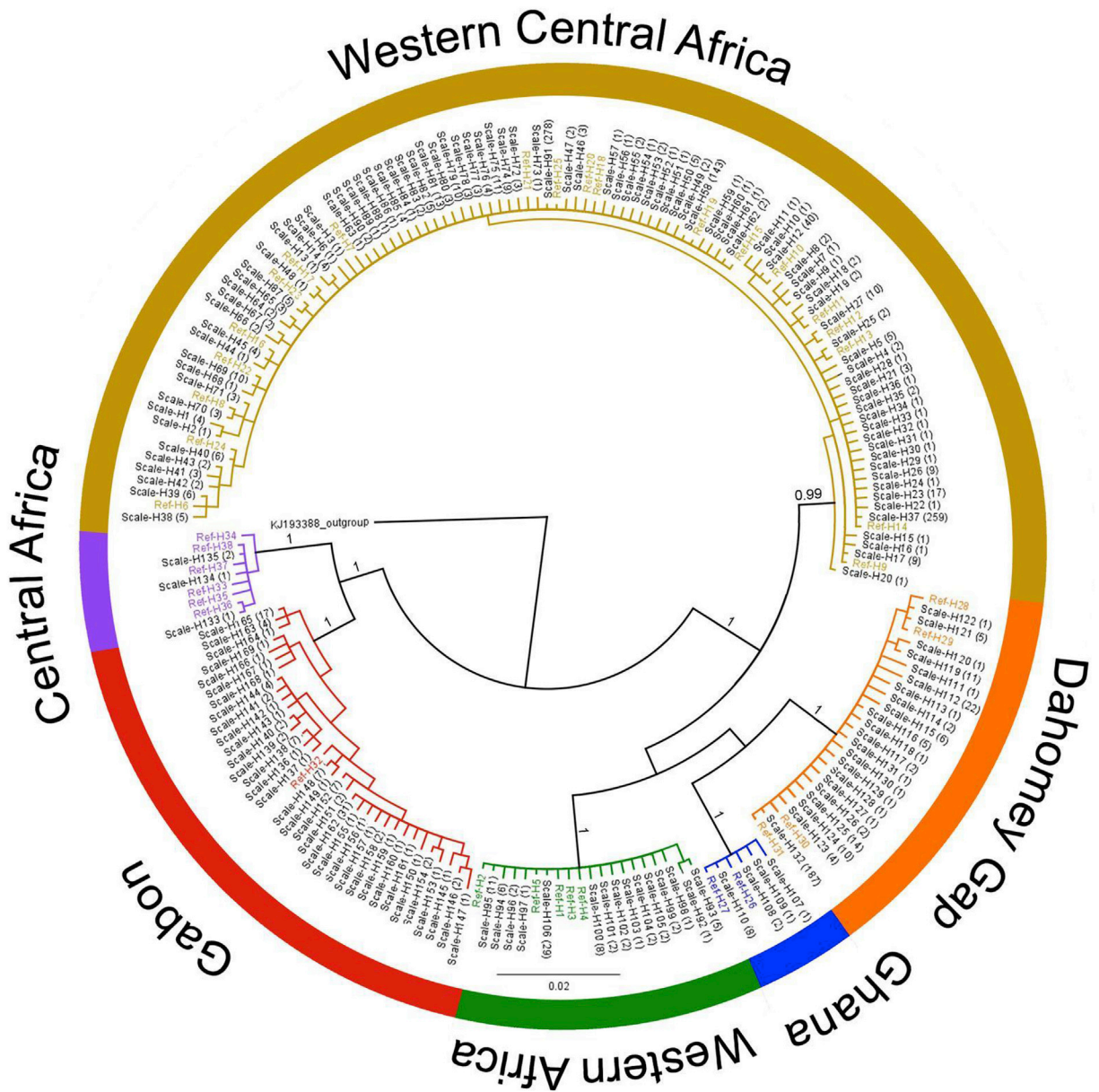


**Fig. 2.** Composition for species (A) and source regions (B) in each seizure with export countries and the year of seizure. Source region naming is based on six phylogeographic lineages of *P. tricuspis* described by Gaubert et al. (2016). Origins are not determined for samples from the other three species or samples not amplified.

Based on our data, *P. tricuspis* is the most commonly traded African pangolin species and this result is consistent with Ingram et al. (2018). However, more studies are needed to fully characterize the trade and assess species composition. Previous studies suggest that *P. tricuspis* is more abundant in Central and West Africa than the other pangolin species, but population declines have been documented (Gaubert et al., 2016; Pietersen et al., 2019). It is commonly found in local and regional bushmeat and traditional medicine markets (Boakye et al., 2016; Fa et al., 2006; Soewu and Sodeinde, 2015), as well as the intercontinental trade from Africa to Asia (Heinrich et al., 2016; Mwale et al., 2017).

*Phataginus tricuspis* was detected together with the other African pangolin species in most seizures, predominantly with *P. tetradactyla*. The range of these two pangolins, together with *S. gigantea*, overlap throughout much of Central and West Africa. Our results suggest that these three species are probably being trafficked together in the range areas. The fourth species (*S. temminckii*) from Eastern and Southern Africa, was only found in a single seizure from Uganda. This species has been recorded in both local and international trade (Challender and Hywood, 2012; Pietersen et al., 2014), and it is not clear why it was under-represented in the Hong Kong seizures. However, lack of overlap in its distribution range with the other three species may influence its occurrence in the present consignments. Further studies are needed to understand how supply chains, trade routes, and law enforcement levels in source and destination countries affect the illegal trade (Challender et al., 2015; Ingram et al., 2018; Shepherd et al., 2017).

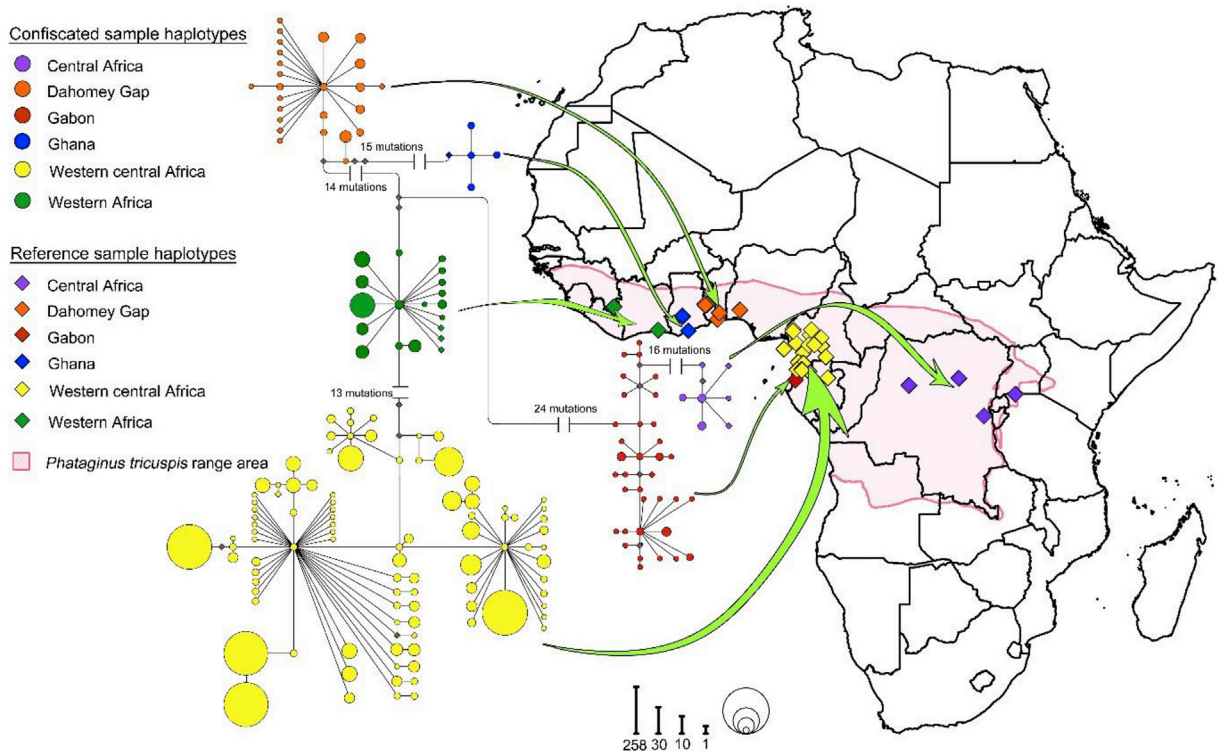
Gaubert et al. (2016) studied *P. tricuspis* across its native geographical range and reported six genetically differentiated lineages. We found all six phylogeographic lineages among the seized samples, suggesting widespread hunting and poaching throughout the areas where reference samples were collected. However, reference samples of the other three African pangolins were geographically limited and more work is required in order to establish reference DNA databases for provenance identification of pangolin seizures, ideally to finer geographic scales (country or population level).



**Fig. 3.** Phylogenetic reconstruction based on 402 bp of the cytochrome *b* gene showing six phylogeographic lineages of *P. tricuspis* found in scale samples. Numbers above branches are Bayesian posterior probability. The reference samples are colored by the six phylogeographic lineages according to Gaubert et al. (2016), and the scale haplotypes are in black color with the number of samples in parentheses. Outgroup taxon is *P. tetradactyla*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The present data suggests that the western central Africa lineage of *P. tricuspis* is a major source of the illegal trade, and origins of the consignments include Cameroon, Equatorial Guinea, and northeastern Gabon where the reference specimens were collected (Gaubert et al., 2016). Western central Africa is among the world's biodiversity hotspots but is also one of the most intensively hunted regions (Fa et al., 2006). A recent study using DNA analysis of seized ivory by Wasser et al. (2015) showed that Africa's major poaching hotspots for forest elephants occur in this area. The number of pangolins hunted in western central Africa has increased dramatically (Ingram et al., 2018, 2019), and more work is needed to understand how local and international demand affects pangolin hunting and illegal trade in this area.

Seizures in Hong Kong suggest that Nigeria and Cameroon play a significant role in the intercontinental illegal pangolin trade as most of the scales in this study were exported from these two countries, and this result is also consistent with findings from global seizure data (Gomez et al., 2016; Heinrich et al., 2017; Ingram et al., 2019). However, the trade routes are complicated and export records are also found in several other African countries, such as Ghana, Kenya and Uganda.



**Fig. 4.** Haplotype networks for the cytochrome *b* gene of *P. tricuspis* clades under the 95% parsimony criterion. The circles and diamonds represent confiscated and reference samples respectively. Each color represents a single clade. Circle size is proportional to sample size. Reference sample locations are from Gaubert et al. (2016). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Our analysis has demonstrated that there is high mtDNA haplotype richness in the seizures from Africa. A more intensive sampling exercise involving two pangolin scale seizures from Southeast Asia only detected 10 to 13 mtDNA haplotypes (Zhang et al., 2015). The genetic data has suggested that small air parcels of pangolin scales from Africa were subsets of large collections because DNA haplotype richness found in small air parcels was comparable to that found in large-volume sea shipments. Pangolins may have been collected over a large geographic area and then sent to a small number of transit or shipment points for inter-continental trade, as supported by our data which has shown that most seizures had multiple phylogeographic lineages of *P. tricuspis*. Similar results were found in pangolin scale seizures from Southeast Asia where different genetic lineages found in a seizure were likely to have been collected from wide geographical areas (Zhang et al., 2015). It is likely that individuals or groups involved in this illegal trade are using different means to smuggle pangolin scales. Our results have important implications for wildlife crime investigation because in many cases, only large-volume or high-value seizures are reported or investigated. However, small-volume seizures also need attention because traders can divide a large collection of pangolin scales into many small parcels posted to the transit and destination countries. Risk profiling with intelligence-assisted approaches is required to enhance detection of illegal wildlife trade and origin points (Heinrich et al., 2019).

Statistical modelling suggests that our sampling has been able to capture the species richness and phylogeographic lineages from the studied seizures. The M1, M2, and M3 estimates of the number of haplotypes were significantly larger than

**Table 1**

Summary statistics for the number of species, phylogeographic lineages and haplotypes compared between observed and modelled data (M1, M2, M3 and regression-based estimate) of the 30 studied seizures.

	Species #	Phylogeographic lineages #	Haplotypes #
Observed	1.87 (0.63)	1.73 (0.58)	16.50 (5.30)
M1	1.88 (0.64)	1.74 (0.59)	20.69 (8.71)*
M2	2.19 (1.03)	1.89 (0.77)	43.73 (22.91)*
M3	2.48 (1.48)*	2.02 (1.02)	89.25 (66.20)*
regression-based estimate	6.03 (19.12)	4.98 (17.34)	46.75 (104.75)

Abbreviations: data shown are Mean (Standard deviation) for observed and modelled data (M1, M2, M3 and regression-based estimate).

\*significant ( $p < 0.05$ ) for t-tests between observed and modelled data.



**Table 2**

Spearman rank correlations (Spearman's rho with p value) between volume of seized scales (natural log transformed) with the number of haplotypes, derived from both observed and modelled data (M1, M2, M3 and regression-based estimate) of the 30 studied seizures.

	observed	M1	M2	M3	regression-based estimate
volume of seized scales	0.13 (0.50)	0.12 (0.54)	0.09 (0.64)	0.14 (0.47)	0.36 (0.05)

observed values, whereas regression-based estimates were not significantly different than observed numbers. From a practical perspective, more work is required to better understand which estimation approach works best under different scenarios, particularly because there were large differences between estimated and observed data in several large-volume seizures. Modelled data suggested large-volume seizures would display larger numbers of haplotypes, and correlations were found between seizure volume and haplotype richness for regression-based estimates, but not for observed data. These results suggest more sampling effort is required for large-volume seizures. There are inherent difficulties in sampling large-volume seizures which contain tons of scales, but unbiased sampling of larger sample sizes is needed in order to develop more appropriate assessments (National Zoological Gardens of South Africa, 2017). Further, the study suggests that the use of statistical modelling can assist in the evaluation of the sampling efforts and the accuracy of the estimates.

The present study highlights the usefulness of DNA analysis to monitor and characterize the trade in pangolin scales, and these results can help guide future law enforcement and trade interventions. DNA samples are required to be taken from large ivory seizures by CITES to facilitate geographic origin analysis (CITES, 2013). Our results suggest that equivalent efforts and procedures applied to pangolin seizures may yield useful information for long-term monitoring and ongoing law enforcement actions.

### Declaration of competing interest

All authors declare no conflict of interest.

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### Appendix G. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gecco.2020.e01119>.

### Appendix



**Fig. 1.** Sample scales from the four African pangolin species (from left to right: *Phataginus tricuspis*, *P. tetradactyla*, *Smutsia gigantea* and *S. temminckii*) found in seizures.



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