DNA Mini-Barcodes, a Potential Weapon for Conservation and Combating Illegal Trade of Pangolin

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DNA Mini-Barcodes, a Potential Weapon for Conservation and Combating Illegal Trade of Pangolin

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Abstract
Background: Smuggling and illegal trade of pangolins and their scales has drastically reduced the wild population of pangolins. Accurate species identification is currently in urgent need as a powerful weapon for combating pangolin smuggling and trade and conserving the already endangered pangolin species.

Aim of the study: To develop an efficient method based on DNA mini-barcodes for accurate pangolin species identification and authentication of processed pangolin scales against the non-target species.

Materials and methods: The primers for amplifying the DNA mini-barcodes were designed based on cytochrome C oxidase subunit I (COI) gene fragments. The mini-barcodes were compared with the two universal barcodes (COI and Cytb) for performance in pangolin species identification by calculating the Kimura-2-parameter (K2P) distance, assessing the clustering dendrogram, and analyzing the BLAST similarity and barcoding gap. The accuracy of the three barcodes was also compared for authentication of pangolin scales against non-target species.

Results: Comparison of the three barcodes showed that the mini-barcode form COI had the highest amplification success rate (100%) and high variable sites (40.0%), with the ratio of mean inter- to intraspecific distance ratio was 25 and a distinct DNA barcoding gap. In the neighbor-joining (NJ) tree constructed based on the mini-barcode regions, each species of the pangolin family formed an obvious clade respectively, and the clades were all separated from those of the non-target species, indicating that the genetic information in the mini-barcode was sufficient for species identification.

Conclusion: The DNA mini-barcodes based on COI gene fragments provide an effective and accurate method for identification of pangolin species and authentication of pangolin scale products.

Keywords
pangolin scales, cytochrome C oxidase subunit I, cytochrome b, mini-barcodes, species identification

Introduction
Pangolins, one of the eight species of the family Manidae that are all on the edge of extinction, have long been over-exploited for their bush meat and scales in Africa and Asia. In traditional Chinese medicine, the scales of Manis pentadactyla is a high-valued crude drug with such therapeutic activities as activating blood circulation, stimulating menstrual flow, eliminating swelling and purulent, dispelling wind and dredging the collaterals (Chinese Pharmacopoeia Commission, 2015). M. pentadactyla used to be a widely distributed species in the south of the Changjiang River, once commonly seen in South China (including Provinces of Guangdong, Guangxi, Hainan, and Taiwan, etc.), but in the past
few decades, the number of wild *M. pentadactyla* had sharply declined, and in as early as 1989, it had been listed as a national second-class protected animal in China and an endangered species in the Red Book of Chinese Endangered Animals (Zhao, 1998). Over the past three decades a drastic increase in smuggling and illegal trade of pangolins and their scales has occurred (Liu et al., 2011; S. B. Wu et al., 2002), and pangolins are now considered the most trafficked wild mammal species world-wide (Challender et al., 2015; Heinrich et al., 2016; F. Yin et al., 2016). In 2020, the International Union for Conservation of Nature (ICUN, 2020) added pangolins to Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (UNEP-WCMC, 2020).

Accurate species identification is essential for pangolin conservation and combating smuggling and illegal trade of pangolin and pangolin scales. Most recently, pangolins were claimed to be a probable host of SARS-CoV-2, the virus that caused the devastating COVID-19 pandemic (Xiao et al., 2020; T. Zhang et al., 2020). The situation of epidemic prevention further highlights the importance of pangolin species identification. Currently, various pangolin-derived products are being traded illegally, including smoked carcasses, chopped meat, scales, scale powder, and even embryos (Nijman et al., 2016; Soewu & Ayodele, 2009; Zhou et al., 2014). The processed scales of pangolin traded in the markets of medicinal materials are extremely difficult to identify; the counterfeited scales from different species, including hoof nails of pigs (*Sus scrofa/S. scrofa domestica*), cattle (*Bos taurus*), yaks (*B. grunniens*), and sheep (*Ovis aries*), to name a few (Dou & Deng, 1993; Wang et al., 2005; Z. F. Zhang & Wu, 2009), further complicate the issue.

The conservation of wild life benefits greatly from the development of various genetic tools (Pierson et al., 2016). In the case of pangolins, the techniques exploiting various molecular markers developed in the past three decades, including restriction fragment length polymorphism (Y. P. Zhang & Shi, 1991), random amplified polymorphic DNA fingerprinting and microsatellite markers (Xing et al., 2013), specific PCR (Y. Yin et al., 2017), and gene sequencing including DNA barcoding (Hsieh et al., 2011; Jia et al., 2014; Li et al., 2019; Mwale et al., 2017; H. R. Zhang et al., 2015, 2020), have all shown great potentials for identifying and tracing pangolins and their products. High throughput sequencing technology and sequencing of mitochondrial genomes of all the eight pangolin species fueled the development of conservation genomics, which enables molecular tracing of pangolins and their possible illegal trade routes (du Toit et al., 2017; Gaubert et al., 2018; Hassanin et al., 2015; Nash et al., 2018; Tan et al., 2016).

So far, studies describing protocols for efficient identification of processed pangolin scales have not been available. The serious DNA degradation in the processed scales, as a result of exposure to high temperature during the sand-scorching or deep-oil frying process, often leads to failure of acquiring standard cytochrome C oxidase subunit I (COI) barcode from these products (Y. Yin et al., 2017), not to mention the whole mitogenome.

DNA mini-barcodes, which are short DNA sequences less than 200 bp in length, offer a promising alternative for identification of processed biological materials with substantial DNA degradation (Hajibabaei et al., 2006; Meusnier et al., 2008). In recent years, successful attempts have been made to apply DNA mini-barcodes in identification of aged specimens, processed animal and plant products, foods and crude drugs (Chen et al., 2018; Labrador et al., 2019; Parveen et al., 2019; Sultana et al., 2018; Y. J. Wu et al., 2018). In this study, we designed a pair of primers based on COI gene fragments to amplify the mini-barcodes for pangolin species. We compared the efficiency of species delimitation using the mini-barcodes and the existing universal DNA barcodes, and verified its validity in the identification of processed pangolin scales.

**Methods**

**Materials**

Samples of *Manis pentadactyla*, *M. javanica*, *M. crassicaudata*, *M. tricuspis* and *M. gigantea* were obtained from the Guangzhou Wildlife Conservation Center and Guangzhou Zhongliang Pharmaceuticals Co., in the form of muscle tissues or scales (Table 1). All the samples were identified by Dr. Zou Jiejian at Guangdong Wildlife Rescue Center. The vouchers were deposited in the Herbal Medicine Museum of Southern Medical University. Eight commercial samples of pangolin scales were purchased from several randomly selected drug stores in different provinces (Table 1).

**Design of Mini-Barcode Primers**

Mitogenome sequences of pangolins and non-target species were downloaded from GenBank (Supplementary file 1) and aligned by MEGA 5.0 (Tamura et al., 2007). Several pairs of primers for mini-barcodes were designed using Primer Premier 5.0 (Zhai et al., 2008) and synthesized by Invitrogen Biotechnology (Shanghai) Co., Ltd., and the primer pair COIB-F/R had been proven to work well. The sequence of the primer pair targeting a 144-bp fragment in cytochrome oxidase I gene (COI) region are listed below:

\[ \text{COIB-F, 5'-AACCTAGCACA TGAGGAGC-3'}; \]

\[ \text{COIB-R, 5'-AACCTAGCACA TGAGGAGC-3'}; \]
DNA Extraction and PCR Amplification

The outer layer of the crude pangolin scales was scraped off, and the inner layer was ground and pulverized into fine powder using a small electric mill; the processed scales were ground directly with a mortar. The genomic DNA was extracted from the muscle tissues and crude scales using Tiangen genomic DNA extraction kit following the manufacturer’s instructions (Li et al., 2019). For DNA extraction from processed pangolin scales, a modified SDS method was used (Cai et al., 2019), which, compared with the conventional SDS extraction method (Xing et al., 2013), used a higher SDS concentration (2%) and a high concentration (0.1%) of collagenase for sample treatment before extraction.
The COI, cytochrome b (Cytb) and mini-barcode regions were amplified with the primers MCOI-F/MCOI-R (Xing et al., 2013), L14841/H15149 (Prado et al., 2007), and COIB-F/COIB-R, respectively. The PCR was implemented using an Applied Biosystems (Gene Company Limited) thermocycler, with 25 μl reaction volumes consisting of 12.5 μl of 2× Taq PCR MasterMix (with dye; Tiangen, Beijing), 1 μl of each primer pair (2.5 mol/L), 2 μl of genomic DNA, and 8.5 μl of ddH2O. For COI barcode and mini-barcode amplification, thermal cycling was performed with an initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. Thermal cycling for Cytb fragment was performed with an initial denaturation at 94°C for 10 min, followed by 35 cycles of 94°C for 30 s, 45°C for 45 s, and 72°C for 30 s, with a final extension at 72°C for 10 min.

DNA Sequencing and Barcode Analysis

The PCR products were sequenced bi-directionally by Invitrogen Biotechnology (Shanghai) Co., Ltd. All the newly acquired sequences were deposited in GenBank (Table 1). Using the software BioEdit and Clustal X (Thompson et al., 1997), the DNA sequences were manually edited and aligned. The Kimura-two-parameter (K2P) distance model (Kimura, 1980), which was determined as the best fit model using the software ModelFinder (Kalyaanamoorthy et al., 2017), was used to calculate the sequence divergences, and the genetic distance were computed using MEGA 5.0 software (Tamura et al., 2007). We also analyzed the average nucleotide frequencies and sequence variations of the three barcode regions using MEGA 5.0 software (Tamura et al., 2007). TaxonDNA software was used to generate the barcoding gap diagram (Meier et al., 2008).

58 records of COI and 66 records of Cytb sequences in the GenBank representing all of the eight Manis species were downloaded. 17 records of COI sequences and 13 records of Cytb sequences of the non-target species were downloaded as well. In total, 75 records of COI sequence and 79 records of Cytb sequence were downloaded. Our check confirmed that most of the downloaded COI sequences were also uploaded in BOLD (Barcode of Life Data System v4, http://v4.boldsystems.org/) (Ratnasingham & Hebert, 2007), and the species designation has then been verified. From those COI fragments, the mini-barcode region was extracted and included in phylogenetic analysis. Neighbor-joining trees based on the K2P distances were constructed using all the COI, Cytb and mini-barcode sequences (including the sequences acquired in this study), and were later confirmed by bootstrapping (1000 replicates) to assign confidence levels to each branch in the tree.

Verification of the Effectiveness of Mini-Barcode

Eight commercial samples of pangolin scales (Table 1) purchased from different local drug stores were analyzed by PCR amplification of the mini-barcode region and sequence analysis of the products, and the sequencing results were queried via BLAST in GenBank and the identification engine in BOLD system.

Results

PCR and Sequencing Efficiency

For all the muscle tissue and crude scale samples, COI, Cytb and the mini-barcode were all successfully amplified and subsequently sequenced. But for processed pangolin scales, only the mini-barcode region could be amplified from all eight samples, in contrast, the COI barcode was amplified from only two (25%) (Figure 1). All the amplicons from the pangolin scales could be successfully sequenced.

Sequence Analysis

The average nucleotide frequencies and sequence variations of the three barcode regions are available from in Table 2. The base composition of the three barcode sequences all showed obvious biases, featuring a lower GC content than AT content. In COI, Cytb and mini-barcode regions, the numbers of variable sites were 261, 144 and 58, respectively. A shorter length of the barcode region was associated with reduced information for species identification. Among the three barcodes, the Cytb barcode, whose length was not the longest, had the highest percentages of variable sites; the mini-barcode, with the shortest barcode length among the three, had higher percentages of variable sites than COI barcode.
Inter- and Intraspecific Variations

Adequate inter- and intraspecific genetic distances are the main criteria for species identification. Using the K2P model, we calculated the genetic distances of COI barcode, Cytb barcode and mini-barcode. For the mini-barcode, the intraspecific genetic distance in *Manis* ranged from 0 to 0.025, with the maximum intraspecific genetic distance found between *M. tricuspis* and *M. pentadactyla*. The interspecific genetic distance ranged from 0.053 to 0.289 (0.204 on average); the minimum interspecific difference was found between *M. culionensis* and *M. javanica*, and the maximum difference between *M. javanica* and *M. tricuspis*. When the non-target species were taken into account, the interspecific genetic distances averaged 0.228 (range 0.048-0.322). We obtained similar results from COI and Cytb barcodes (Supplementary file 2 and Table 3).

The mean interspecific/intraspecific distance ratio was 27 for COI barcode, 17 for Cytb barcode and 25 for mini-barcode. All the three barcodes had sufficiently large minimum interspecific genetic distances compared to their respective maximum intraspecific genetic distances, and thus met the criterion for a DNA barcode, which is expected to have a minimum interspecific/max-imum intraspecific genetic distance ratio >10 (Hebert et al, 2003). But for the Cytb barcode, the interspecific distance (range 0.049-0.284) was not completely separated from the intraspecific distance (range 0-0.054) and contained overlapping areas with the latter.

DNA Barcoding Gap Assessment

For an eligible barcode, the interspecific genetic variation detected should be significantly greater than the intraspecific genetic variation to allow for a sufficient distance between them, known as the barcoding gap. Ideally, the smaller values representing intraspecific variation are distributed on the left side of the histogram, while larger values for the interspecific variation on the right side. In this study, we computed the barcoding gaps of the three barcodes using TaxonDNA software. For both mini-barcode and COI (Figure 2a and c), a clear barcoding gap was observed, with the values for intraspecific variation occurring mostly on the smaller side (left) and those for interspecific variation mostly on the larger side (right). For Cytb barcodes (Figure 2b), the pairwise intraspecific and interspecies genetic distances showed partial overlap.

Species Identification Based on Neighbor-Joining Tree

In addition to the mini-barcode sequences obtained in this study, we also downloaded the COI gene sequences of other pangolin species and the common non-target species from GenBank. Using MEGA 5.0 software, a neighbor-joining tree based on the mini-barcodes was constructed (Figure 3). Each species formed a monophyletic clade with a high bootstrap support in the tree, although the clades of the counterfeit species nested in those of *Manis* species. The topological structure of COI and CytB NJ tree was similar, with all the *Manis* species divided into two groups: one consisting of Asian species, including *M. pentadactyla*, *M. crassicaudata*, *M. javanica* and *M. culionensis*, and the other of the African species *M. tricuspis*, *M. tetradactyla*, *M. temminckii* and *M. gigantea*. These two groups were distinctly separated from the non-target species groups.

### Table 2. Average Nucleotide Frequencies and Sequences Variation of the 3 Barcode Regions.

| Barcode    | Length | A%   | T%   | G%   | C%   | Insertion/deletion | Variable sites % | Singleton variable sites |
|------------|--------|------|------|------|------|--------------------|------------------|-------------------------|
| Mini-barcode | 145    | 29.9 | 33.1 | 10.9 | 26.1 | 0                  | 40.0             | 1                       |
| COI        | 658    | 26.3 | 30.1 | 17.0 | 26.6 | 0                  | 39.7             | 6                       |
| Cytb       | 307    | 29.4 | 28.7 | 15.5 | 26.4 | 0                  | 46.9             | 3                       |

### Table 3. The Genetic Distances of Pangolins Measured Using the 3 DNA Barcodes.

| Barcode                        | Interspecific distance | Average interspecific distance | Intraspecific distance | Average intraspecific distance |
|-------------------------------|------------------------|-------------------------------|------------------------|-------------------------------|
| *Manis* species only          | Mini-barcode 0.053–0.289 | 0.204 | 0–0.025 | 0.009 |
| COI                           | 0.044–0.266 | 0.199 | 0.001–0.031 | 0.012 |
| Cytb                          | 0.049–0.284 | 0.192 | 0–0.054 | 0.020 |
| *Manis* and non-target species| Mini-barcode 0.048–0.322 | 0.228 | 0–0.025 | 0.009 |
| COI                           | 0.044–0.266 | 0.213 | 0–0.031 | 0.008 |
| Cytb                          | 0.049–0.312 | 0.220 | 0–0.054 | 0.013 |
Identification of Commercial Pangolin Scales Using Mini-Barcode

PCR amplification of the mini-barcodes from the eight randomly purchased drug samples of pangolin scales, either crude or processed, all yielded a positive band of 144 bp (Figure 1), and their sequences were clearly identified at the species level with the BLAST system (Altschul et al.). Each sample showed a maximum identity of 100% in the mini-barcode region to the matched species (Table 4). The results showed that only sample C5 was authentically derived from *M. pentadactyla*, and all the other samples were non-target species derived from *M. javanica*.

Discussion

Pangolins are considered to be the most trafficked mammals on earth, and the protection of pangolins has been upgraded to the highest level globally. As the latest measure in force, the scale of *Manis pentadactyla* has been removed from Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2015). Yet, this measure is not expected to completely eliminate the illegal trade of pangolins and related products. There is still a need for continued regulation and enforcement. Accurate species identification technology is one of the basic work involved.

Many molecular markers have been shown to be effective for monitoring pangolin trade, such as COI, Cytb, and D-loop (Hsieh et al., 2011; Jia et al., 2014; H. R. Zhang et al., 2015). Our work further confirmed the applicability of COI and Cytb barcodes in authentication of crude pangolin scales, for which COI barcode showed a slight advantage over Cytb barcode. For Cytb barcode, the intraspecific and interspecific distances overlapped a little in the barcoding gap diagram, and within the phylogenetic tree based on Cytb sequences, a sequence of *Manis tetradactyla* (NC_004027) (Arnason et al, 2002) was noted to nest in those of *M. tricuspis*. But there’s also a possibility that this sequence might be based on a misidentification, because searching of COI sequence from same sample in BOLD system suggested its probable origin of *Manis tricuspis*. Compared with the more conventional COI and Cytb barcodes, the DNA mini-barcode showed a comparable performance in differentiating *Manis* species. In spite of a shorter barcode length, which is associated with an inevitable loss of some genetic information (Fu et al., 2010), the mini-barcode region provided sufficient information sites in this limited length (144 bp) to produce a clear barcoding gap between the intraspecific and interspecific distances (2.003 and 0.002, respectively). In the phylogenetic tree based on the mini-barcodes, although the clades of the counterfeit species were dispersed among those of *Manis* species, each species had a clearly
separated clade, implying a sufficient capacity of mini-barcodes for species identification. In addition, the BLAST similarities of these mini-barcodes in GenBank and BOLD system reached 100%, higher than that of COI and Cytb barcodes.

Our results demonstrate good performance of DNA mini-barcodes for identifying processed pangolin scales. The acquisition of the target gene fragments with a length exceeding 200 bp (such as COI and Cytb) can often be difficult from highly processed biological materials with substantial DNA degradation (Ye, 2017). As the mini-barcode system requires to amplification of a short DNA fragment of about 150 bp (Ma, 2013), the success rate of obtaining the target amplicons can be much higher as compared with other barcoding systems. Moreover, a proper method to improve DNA extraction from deeply processed materials is necessary (C. Zhao et al., 2020). Previously, we established a method for DNA extraction from processed pangolin scales, which facilitated the identification of the processed samples using mini-barcodes (Cai et al., 2019). In this study, we successfully extracted genomic DNA from eight commercial pangolin scale samples, including six processed samples and two crude ones. COI fragments were amplified only from the two crude scale samples but from none of the processed scales. In contrast, the mini-barcode region was amplified from all the samples, and the subsequent sequencing and species assignment through blasting in GenBank or in BOLD system was achieved.

In conclusion, the mini-barcodes amplified with the primers COIB-F/COIB-R were shown to effectively differentiate the eight pangolin species and identify their counterfeits. The mini-barcodes are also capable of accurately identifying the processed pangolin scales. This work represents the first successful attempt for identification of processed pangolin scales using DNA mini-barcodes, which are expected to show great potential as a powerful tool in conservation of wild populations of pangolins.

**Implications for Conservation**

Even though the trade and usage of pangolins and related products have long been prohibited worldwide, smuggling and illegal trade of pangolin products are still occurring. Accurate and rapid identification of pangolin products is essential for combating these illegal activities. The authentication of processed pangolin scales commonly used for medicinal purposes remains difficult due to serious DNA degradation in the course of processing involving high temperature exposures. We demonstrate herein that the properly selected mini-barcode fragments enable accurate identification of processed scales derived from pangolins and other animal species.
Table 4. Identification Results of Purchased Samples of Pangolin Scales With Mini-Barcode.

| Sample no. | Collecting sites | Property    | Identified by BLAST   | Sequence similarity (%) |
|------------|------------------|-------------|-----------------------|-------------------------|
| C1         | Shandong         | Crude       | Manis javanica        | 100%                    |
| C2         | Zhejiang         | Processed   | Manis javanica        | 100%                    |
| C3         | Hubei            | Processed   | Manis javanica        | 100%                    |
| C4         | Hunan            | Processed   | Manis javanica        | 100%                    |
| C5         | Guangdong        | Crude       | Manis pentadactyla    | 100%                    |
| C6         | Sichuan          | Processed   | Manis javanica        | 100%                    |
| C7         | Shandong         | Processed   | Manis javanica        | 100%                    |
| C8         | Yunan            | Processed   | Manis javanica        | 100%                    |

and can potentially be used as a powerful weapon for combating smuggling and illegal trade of both crude and processed pangolin scales.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material
Supplementary material for this article is available online.

References
Altschul S. F. (2012). Basic local alignment search tool (BLAST). Journal of Molecular Biology, 215(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2
Arnason U., Adegoke J. A., Bodin K., Born E. W., Esa Y. B., Gullberg A., Nilsson M., Short R. V., Xu X., & Janke A. (2002). Mammalian mitogenomic relationships and the root of the eutherian tree. Proceedings of the National Academy of Sciences of the United States of America, 99(12), 8151–8156. https://doi.org/10.1073/pnas.102164299
Cai, X., Ye, H. T., Tian, E. W., Li, F., Li, C., Yang, Y. J., Liu, C. S., & Chao, Z. (2019). Successful and efficient DNA extraction from processed pangolin scales. Sains Malaysiana, 48(3), 555–559. https://doi.org/10.17576/jsm-2019-4803-07
Challender, D. W. S., Harrop, S. R., & MacMillan, D. C. (2015). Understanding markets to conserve trade-threatened species in CITES. Biological Conservation, 187, 249–259. https://doi.org/10.1016/j.biocon.2015.04.015
Chen, M. Y., Han, X., Ma, J., Liu, X. Y., Li, M., Lv, X. N., Lu, H. S., Ren, G. X., & Liu, C. S. (2018). Identification of Plastrum testudinis used in traditional medicine with DNA mini-barcodes. Revista Brasileira De Farmacognosia-Brazilian Journal of Pharmacognosy, 28(3), 267–272. https://doi.org/10.1016/j.rjbfp.2018.04.008
Chinese Pharmacopoeia Commission. (2015). The pharmacopoeia of the People’s Republic of China 2015 edition (Vol. 1, p. 268). Medical Science and Technology Press.
Dou, Y. Y., & Deng, J. X. (1993). Identify the authenticity of Pangolin. Lishizhen Medicine and Materia Medica Research 4(1), 25.
du Toit, Z., du Plessis, M., Dalton, D. L., Jansen, R., Grobler, J. P., & Kotze, A. (2017). Mitochondrial genomes of African pangolins and insights into evolutionary patterns and phylogeny of the family Manidae. BMC Genomics, 18(1), 746. https://doi.org/10.1186/s12864-017-4140-5
Fu, M., Peng, J. J., Wang, Y., Yu, D. M., Wang, L. L., & Zhang, Y. S. (2010). Application and analysis of DNA barcoding. Journal of Henan Normal University (Natural Science), 38(4), 118–122. https://doi.org/10.16366/j.cnki.1000-2367.2010.04.020
Gaubert, P., Antunes, A., Meng, H., Miao, L., Peigne, S., Justy, F., Njokouk, F., Dufour, S., Danquah, E., Alakahoons, J., Verheyen, E., Stanley, W. T., O’Brien, S. J., Johnson, W. E., & Luo, S. J. (2018). The complete phylogeny of pangolins: Scaling up resources for the molecular tracing of the most trafficked mammals on earth. Journal of Heredity, 109(4), 347–359. https://doi.org/10.1093/1093/jhered/esx097
Hajibabaei, M., Smith, M. A., Janzen, D. H., Rodriguez, J. J., Whitfield, J. B., & Hebert, P. D. N. (2006). A minimalist barcode can identify a specimen whose DNA is degraded. Molecular Ecology Notes, 6(4), 959–964. https://doi.org/10.1111/j.1471-8286.2006.01470.x
Hassanin, A., Hugot, J. P., & van Vuuren, B. J. (2015). Comparison of mitochondrial genome sequences of pangolins (Mammalia, Pholidota). Comptes Rendus Biologies, 338(4), 260–265. https://doi.org/10.1016/j.crvi.2015.02.003
Hebert, P. D., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003). Biological identifications through DNA barcodes. Proceedings of the Royal Society B: Biological Sciences, 270(1512), 313–321. https://doi.org/10.1098/rspb.2002.2218
Heinrich, S., Wittmann, T. A., Prowse, T. A. A., Ross, J. V., Delean, S., Shepherd, C. R., & Cassey, P. (2016). Where did all the pangolins go international CITES trade in pangolin
species. *Global Ecology and Conservation, 8*, 241–253. https://doi.org/10.1016/j.gecco.2016.09.007

Hsieh, H. M., Lee, J. C., Wu, J. H., Chen, C. A., Chen, Y. J., Wang, G. B., Chin, S. C., Wang, L. C., Linacre, A., & Tsai, L. C. (2011). Establishing the pangolin mitochondrial D-loop sequences from the confiscated scales. *Forensic Science International: Genetics, 5*(4), 303–307. https://doi.org/10.1016/j.fsigen.2010.06.003

International Union for Conservation of Nature (IUCN). (2020, July 9). *The IUCN red list of threatened species*. https://www.iucnredlist.org

Jia, J., Zhang, H. Y., Chen, J., Liu, D., Yao, H., Qian, Q. N., & Meusnier, I., Singer, G. A. C., Landry, J. F., Hickey, D. A., Mwale, M., Dalton, D. L., Jansen, R., De Bruyn, M., Meier, R., Zhang, G. Y., & Ali, F. (2008). The use of mean Xie et al. (2012). *Research progress in DNA mini-barcoding and minibarcode amplification*. *Philippine sardine products using hotshot DNA extraction and metabarcoding*. *Journal of Agricultural Catastrophology, 6* (7), 58–60. https://doi.org/10.1016/j.jca.2013.06.021

Kimura, M. (1980). A simple method for estimating evolution- ary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution, 16*(2), 111–120. https://doi.org/10.1007/bf01731581

Labrador, K., Agmata, A., Palermo, J. D., Follante, J., & Pante, M. J. (2019). Authentication of processed Philippine sardine products using hotspot DNA extraction and minibarcode amplification. *Food Control, 98*, 150–155. https://doi.org/10.1016/j.foodcont.2018.11.027

Li, C., Xie, X., Cai, X., Zhang, Q., Tian, E., & Chao, Z. (2019). DNA barcode of pangolins and its application in identification of *Manis* squama commodities. *Modern Chinese Medicine, 21*(9), 1221–1228. https://doi.org/10.13313/j.issn.1673-4890.20190217002

Liu, X. Q., Peng, J. J., Gao, S. F., Yu, D. M., Gao, L. F., Wang, L. L., Hu, S. J., & Fu, M. L. (2011). An overview of pangolin smuggling trade, species identification and morphological comparison. *Forest Science and Technology 54*(5), 11–14. https://doi.org/10.13456/j.cnki.lykt.2011.05.029

Ma, L. (2013). Research progress in DNA mini-barcoding and meta-barcoding. *Journal of Agricultural Catastrophology, 3* (6), 58–60+63. https://doi.org/10.19383/j.cnki.nyzhjy.2013.06.021

Meier, R., Zhang, G. Y., & Ali, F. (2008). The use of mean instead of smallest interspecific distances exaggerates the size of the “Barcoding Gap” and leads to misidentification. *Systematic Biology, 57*(5), 809–813. https://doi.org/10.1080/10635150802406343

Meusnier, I., Singer, G. A. C., Landry, J. F., Hickey, D. A., Hebert, P. D. N., & Hajibabaei, M. (2008). A universal DNA mini-barcode for biodiversity analysis. *BMC Genomics, 9*, Article 214. https://doi.org/10.1186/1471-2164-9-214

Mwale, M., Dalton, D. L., Jansen, R., De Bruyn, M., Pietersen, D., Mokgokong, P. S., & Kotze, A. (2017). Forensic application of DNA barcoding for identification of illegally traded African pangolin scales. *Genome, 60*(3), 272–284. https://doi.org/10.1139/gen-2016-0144

Nash, H. C., Wirdateti, Low, G. W., Choo, S. W., Chong, J. L., Semiaidi, G., Hari, R., Sulaiman, M. H., Turvey, S. T., Evans, T. A., & Rheindt, F. E. (2018). Conservation genomics reveals possible illegal trade routes and admixture across pangolin lineages in Southeast Asia. *Conservation Genetics, 19*(5), 1083–1095. https://doi.org/10.1007/s10592-018-1080-9

Nijman, V., Zhang, M. X., & Shepherd, C. R. (2016). Pangolin trade in the Mong La wildlife market and the role of Myanmar in the smuggling of pangolins into China. *Global Ecology and Conservation, 5*, 118–126. https://doi.org/10.1016/j.gecco.2015.12.003

Parveen, I., Techen, N., & Khan, I. A. (2019). Identification of species in the aromatic spice family Apiaceae using DNA Mini-barcodes. *Planta Medica, 85*(2), 139–144. https://doi.org/10.1055/a-0664-0947

Pierson, J. C., Coates, D. J., Oostermeijer, J. G. B., Beissinger, S. R., Bragg, J. G., Sunnucks, P., Schumaker, N. H., & Young, A. G. (2016). Genetic factors in threatened species recovery plans on three continents. *Frontiers in Ecology and the Environment, 14*(8), 433–440. https://doi.org/10.1002/fee.1323

Prado, M., Calo-Mata, P., Villa, T. G., Cepeda, A., & Barros-Velazquez, J. (2007). Co-amplification and sequencing of a cytochrome B fragment affecting the identification of cattle in PCR-RFLP food authentication studies. *Food Chemistry, 105*(1), 436–442. https://doi.org/10.1016/j.foodchem.2007.03.045

Ratnasingham, S., & Hebert, P. D. N. (2007). BOLD: The barcode of life data system (www.barcodinglife.org). *Molecular Ecology Notes, 7*(3), 355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x

Soewu, D. A., & Ayodele, I. A. (2009). Utilisation of pangolin (Manis ssp) in traditional Yorubic medicine in Ijebu province, Ogun State, Nigeria. *Journal of Ethnobiology and Ethnomedicine, 5*(1). Article 39. https://doi.org/10.1186/1746-4269-5-39

Sultana, S., Ali, M. E., Hossain, M. A. M., Asing, Naquiah, N., & Zaidul, I. S. M. (2018). Universal mini COI barcode for the identification of fish species in processed products. *Food Research International, 105*, 19–28. https://doi.org/10.1016/j.foodres.2017.10.065

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution, 24*(8), 1596–1599. https://doi.org/10.1093/molbev/msm092

Tan, T. K., Tan, K. Y., Hari, R., Yusoff, A. M., Wong, G. J., Siow, C. C., Mutha, N. V. R., Rayko, M., Komissarov, A., Dobrynin, P., Krasheninnikova, K., Tamazian, G., Paterson, I. C., Warren, W. C., Johnson, W. E., O’Brien, S. J., & Choo, S. W. (2016). PGD: A pangolin genome hub and admixture across pangolin lineages in Southeast Asia. *Database: The Journal of Biological Databases and Curation, 2017*, Article baw063. https://doi.org/10.1093/database/baw063

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment...
aided by quality analysis tools. *Nucleic Acids Research*, 25(24), 4876–4882. https://doi.org/10.1093/nar/25.24.4876

UNEP-WCMC. (2020, October 20). The checklist of CITES species website. http://checklist.cites.org

Wang, Z., Wen, H. L., Huang, G., Wu, L. H., & Xu, R. (2005). The standardization of adulterated pangolin. *Guiding Journal of Traditional Chinese Medicine and Pharmacology*, 11(8), 59–60 + 89. https://doi.org/10.13862/j.cnki.cn43-1446/r.2005.08.030

Wu, S. B., Ma, G. Z., Tang, M., Chen, H., & Liu, N. F. (2002). The status and conservation strategy of pangolin resource in China. *Journal of Natural Resources*, 17(2), 174–180. https://doi.org/10.3321/j.issn:1000-3037.2002.02.008

Wu, Y. J., Li, M. G., Yang, Y. G., Jiang, L., Liu, M. C., Wang, B., & Wang, Y. C. (2018). Authentication of small berry fruit in fruit products by DNA barcoding method. *Journal of Food Science*, 83(6), 1494–1504. https://doi.org/10.1111/1750-3841.14177

Xiao, K. P., Zhai, J. Q., Feng, Y. Y., Zhou, N., Zhang, X., Zou, J. J., Li, N., Guo, Y. Q., Li, X. B., Shen, X. J., Zhang, Z. P., Shu, F.-f., Huang, W. Y., Li, Y., Zhang, Z., Chen, R. A., Wu, Y. J., Peng, S. M., Huang, M., Shen, Y. (2020). Isolation of SARS-CoV-2-related coronavirus from Malayan pangolins. *Nature*, 583(7815), 286–289. https://doi.org/10.1038/s41586-020-2313-x

Xing, Y. L., Peng, J. J., Hu, J. H., Yu, D. M., Zhang, L., & Yu, B. C. (2013). Extraction and amplification of DNA from pangolin specimen and scales. *Chinese Journal of Zoology*, 48(1), 49–57. https://doi.org/10.13859/j.cjz.2013.01.012

Ye, J. (2017). Research on molecular identification methods of common squid species in Family Ommastrephidae [MS dissertation]. Zhejiang Gongshang University.

Yin, F., Lu, L. L., Meng, M., & Liu, D. Z. (2016). Trade and conservation of pangolin. *Chinese Journal of Wildlife*, 37(2), 157–161. https://doi.org/10.19711/j.cnki.issn2310-1490.2016.02.016

Yin, Y., Liu, X., Wang, B., Gao, L. H., & Zhang, X. N. (2017). DNA molecular identification of *Manis pentadactyla*. *China Journal of Chinese Materia Medica*, 42(11), 2078–2084. https://doi.org/10.19540/j.cnki.cjcmmm.2017.0092

Zhai, Z. H., Chen, X. N., & Wang, J. (2008). Primer design with primer Premier 5.0. *Northwest Medical Education*, 16(4), 695–698. https://doi.org/10.3969/j.issn.1006-2769.2008.04.042

Zhang, H. R., Miller, M. P., Yang, F., Lai, K. W., & Fischer, G. A. (2020). Genetic identification of African pangolins and their origin in illegal trade. *Global Ecology and Conservation*, 23, Article e01119. https://doi.org/10.1016/j.gecco.2020.e01119

Zhang, H. R., Miller, M. P., Yang, F., Chan, H. K., Gaubert, P., Ades, G., & Fischer, G. A. (2015). Molecular tracing of confiscated pangolin scales for conservation and illegal trade monitoring in Southeast Asia. *Global Ecology and Conservation*, 4, 414–422. https://doi.org/10.1016/j.gecco.2015.08.002

Zhang, T., Wu, Q. F., & Zhang, Z. G. (2020). Probable pangolin origin of SARS-CoV-2 associated with the COVID-19 outbreak. *Current Bioinformatics*, 30(7), 1346.e1342–1351. e1342. https://doi.org/10.1016/j.j.cub.2020.03.022

Zhang, Y. P., & Shi, L. M. (1991). Genetic diversity in the Chinese pangolin (*Manis pentadactyla*): Inferred from restriction enzyme analysis of mitochondrial DNAs. *Biochemical Genetics*, 29(9-10), 501–508. https://doi.org/10.1007/BF02399690

Zhang, Z. F., & Wu, Z. C. (2009). Yang SC’s experience in the identification of pangolins and processed products. *World Health Digest*, 6(13), 176–177. https://doi.org/10.3969/j.issn.1672-5085.2009.13.200

Zhao, E. (1998). *China red data book of endangered animals—Amphibia & Reptilia*. Science Press.

Zhao, C., Qiao, X., Shao, Q., Hassan, M., & Ma, Z. (2020). Evolution of lignin chemical structure during bioethanol production process and its inhibition to enzymatic hydrolysis. *Energy Fuels*, 34(5), 5938–5947. https://doi.org/10.1021/acs.energyfuels.0c00293

Zhou, Z. M., Zhou, Y. B., Newman, C., & Macdonald, D. W. (2014). Scaling up pangolin protection in China. *Frontiers in Ecology and the Environment*, 12(2), 97–98. https://doi.org/10.1890/14.Wb.001