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Establishing the pangolin mitochondrial D-loop sequences from the confiscated scales

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ABSTRACT

Pangolin scales are encountered in traditional East Asian medicines (TEAM) and the ever increasing demand for these scales has escalated the decline in the numbers of these mammals. The identification of protected pangolin species is necessary to enforce international and national legislation as well as assist with conservation measures. There is limited morphological feature on a pangolin scale thus requiring DNA analysis as a means of identification. We report on the isolation of DNA from pangolin scales and a strategy for obtaining the full length of the mitochondrial D-loop, being 1159 bp. Primer sets creating five overlapping amplicons were designed to amplify sections of this mitochondrial DNA locus. DNA from the blood stain of nineteen Formosan pangolins (*Manis pentadactyla pentadactyla*) along with 145 scale samples that were suspected to have come from pangolins, was amplified and sequenced; leading to a total of 91 D-loop sequences being obtained. The 19 Formosan pangolin sequences produced 5 haplotypes and 72 of the 145 seized scales provided useable sequence classified as a further 38 haplotypes. The D-loop sequences from those scales suspected to be from a pangolin had a higher similarity to any of the 19 samples taken from *M. p. pentadactyla* compared to a D-loop sequence from *Manis tetradactyla* (the only pangolin D-loop sequence in GenBank, NC_004027). These 43 haplotypes were used to establish a local database for the D-loop sequence of pangolins and add to the data of *Manis* sp. held on GenBank. The PCR amplification strategy development in this study could be used in forensic DNA identification of scales suspected to be from protected pangolin species.

1. Introduction

There are eight extant species of pangolin, four of which are distributed within Africa (*Manis tricuspis*, *M. gigantea*, *M. tetradactyla* and *M. temminckii*) and four over Asia (*M. pentadactyla*, *M. culionensis*, *M. crassicaudata* and *M. javanica*) [1]. All eight species are listed on one of the three appendices of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The numbers of all eight species are in decline due in part to their tissues used in food products and their scales featuring in traditional East Asian medicines (TEAM). The Formosan pangolin (*M. pentadactyla pentadactyla*) is the only endemic subspecies in Taiwan and, as their numbers are declining, there is an increase in the illegal importation into Taiwan of pangolin scales for TEAM preparations. The limited morphological features on pangolin scales make it difficult to positively identify a protected species visually, raising the need for a DNA test. The DNA within keratinized pangolin scales is most likely to be degraded and in trace amounts. DNA extraction of the keratinized hair and the successful analysis of trace levels DNA has been reported previously, such as from animal hairs [2–6], rhino horns [7], ivory samples [8], turtle shells [9], ancient or degraded bones [10–12], handled objects [13] and burnt stubs [14].

Previous studies involving pangolin have focused on ecology [15], behaviour [16], physiology [17,18] and comparative anatomy [19]. Some limited studies using pangolin DNA focused on the evolution and phylogeny of mammals in general [20–25] and comparative genome studies [26]. Currently there are little data on the genetic diversity of pangolin. In 1991, Zhang and Shi reported

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the genetic diversity of *M. pentadactyla* based on the partial sequence of the cytochrome b (cyt *b*) gene by restriction enzyme analysis [27], and in 2007, Luo et al. developed dinucleotide microsatellite markers within the nuclear genome of the Malayan pangolin (*M. javanica*) [28]. These methods do not result in the unambiguous identification of the protect species, particularly when applied to keratinized samples such as panglin scale where nuclear DNA testing may not possible. Currently registered on GenBank is one complete pangolin mitochondrial genome from *M. tetradactyla* (accession number NC_004027) and full length or partial gene sequences including: the partial cyt *b* of *M. p. pentadactyla* (EU057624–EU057637 and AJ304500), 12S of *Manis* sp. (AY012154, AF107220 and U61079) and 16S of *Manis* sp. (AY011188, AF107226 and U97340).

The mtDNA D-loop region of other mammalian species has been used in phylogenetic and population studies [29–31] and in forensic science studies [32–34]. The D-loop sequence offers a chance of identification of unknown samples as originating from a pangolin, although there is currently only the one sequence on the GenBank (NC_004027). We report on strategy to obtain full D-loop sequence data from highly degraded DNA obtained from seized scales and a comparison of the resulting data from 19 known Formosan pangolins.

2. Materials and methods

2.1. Sample collections and DNA extraction

A total number of 145 scales, suspected to come from pangolins, were provided by the Council of Agriculture (COA) and the National Museum of Natural Science, Taiwan. Nineteen blood samples of *M. p. pentadactyla* were provided by Taipei Zoo as reference samples. A sample taken from a pangolin corpse, found on a Taiwan mountain, was selected as a blind trial sample.

DNA extraction from pangolin scales was performed using the Extractor FM Kit (Wako Pure Chemical, Osaka, Japan); this kit has been used on forensic samples such as hair shafts and bone [5,6]. After pulverizing the pangolin scale with a sterilized blender, approximately 30 mg of pangolin scale powder was suspended in extraction buffer (190 μL lysis solution, 10 μL enzyme-activated reagent solution and 10 μL 100 μg/μL Proteinase K) and incubated at 56 °C overnight. A negative control using 30 μL of ddH_2O in place of the pangolin scale was co-extracted with the pangolin scale samples. After DNA precipitation and a washing procedure, the dried DNA pellet was dissolved in 30 μL of ddH_2O. DNA extraction of blood samples from the reference material and the muscle tissue of the pangolin corpse were extracted by using the Blood and Tissue Genomic Mini Kit (Viogene, Taiwan).

2.2. Primer design

The eight primer sequences and the primer pairs designed to produce six amplicons are shown in Tables 1 and 2. The priming sites and predicted amplicon size are shown in Fig. 1. Primers L15997uni and H600uni were designed to amplify the full length of the D-loop and were designed according to the sequences upstream and downstream of the D-loop region of *M. tetradactyla* (Accession no. NC_004027). The other six primers were designed according to the consensus sequences of 19 reference samples (*M. p. pentadactyla*) generated in this study and the D-loop region of *M. tetradactyla* (Accession no. NC_004027).

Table 1
The primer sequences used in this study.

Primer name	Primer sequence (5′–3′)
L15997uni	AGCCCCCAAAGCTGATATTCT
panDL15825	CGTGTCCCTCTTCTCGCTCC
panDL15943	CTTAAATAAGACATCTCGATGG
panDL16281	GTACTAAACATCTTGTCAAACC
panDH15827	CCCACAGTCTATATGGGCC
panDH15972	AGGGCATGACACCACAGTTATG
panDH16260	GGTTTGACAAGATGTTTAGTAC
H600uni	CATTTTCAGTGCTTTGCTTT

2.3. PCR amplification of D-loop region and DNA sequencing

PCR amplifications were performed in 50 μL of reaction mixture, which contained 5 μL of genomic DNA, reaction buffer (1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% (w/v) gelatine and 0.1% TritonX-100), 0.15 μM each of primers, 100 μM dNTP and 1.25 units of VioTaq DNA polymerase (Viogene, Taiwan). The amplification was conducted in a GeneAmp PCR System 9700 (Applied Biosystems, Foster, CA, USA) and with the following conditions: 30–40 cycles of 94 °C for 60 s, 52.2–61.2 °C for 45 s and 72 °C for 30–60 s. The detailed amplification condition for each primer set is shown in Table 2. PCR products were checked on a 2% agarose gel, purified with the PCR-M™ Clean Up System (Viogene), and were sequenced using the forward and reverse primers in conjunction with the BigDye™ Terminator Kit (ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems). The cycle sequencing products were purified by ethanol precipitation, separated using a POP-7™ polymer (Applied Biosystems) and detected using an ABI 3730 DNA Analyzer.

2.4. Sequence analysis and establishment of a DNA database

The D-loop sequences of all the collected samples were aligned and the pangolin DNA database was established using the BioEdit software [35].

3. Results and discussion

3.1. Establishment of the D-loop sequence for the Formosan pangolin mtDNA

An amplicon of approximately 1240 bp was generated when using the outer primers (L15997uni and H600uni) to amplify the full length of the D-loop from the nineteen reference samples. The complete sequence data for both strands of these amplicons were obtained successfully. After comparison with the mtDNA sequence of *M. tetradactyla* and excluding the upstream and downstream sequences of the D-loop region, the 1159 bp

Table 2
The primer pairs and the PCR conditions used in this study to amplify the D-loop region.

Fragment	Primer pair	Size (bp)	Annealing temp	Extension time (s)	Cycle number
A	L15997uni/H600uni	1240	58.3 °C	60	40
B1	L15997uni/panDH15972	625	61.2 °C	45	35
B2	panDL15943/H600uni	689	52.2 °C	45	35
C1	L15997uni/panDH15827	477	61.2 °C	30	30
C2	panDL15825/panDH16260	478	61.2 °C	30	30
C3	panDL16281/H600uni	351	58.3 °C	30	30