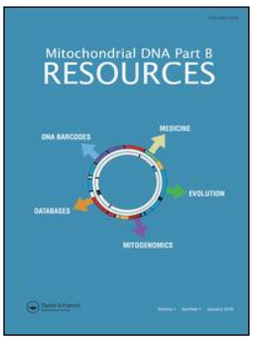

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Determining species identity from confiscated pangolin remains using DNA barcoding

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ABSTRACT

Illegal wildlife trade is one of the key threats to biodiversity. A requisite in combating illegal wildlife trade is through effective and efficient identification of confiscated wildlife or wildlife remains. This can be done through DNA barcoding. In this study, DNA barcoding was employed on several cases of poaching in the Philippines involving 85 unidentified pangolin remains. Of these, 73 specimens confiscated from Palawan were identified as the Palawan endemic *Manis culionensis*, but no deep divergences were observed, suggesting that the samples originated from a single locality. The other 12 individuals, which were part of a large haul of pangolin carcasses recovered from a foreign fishing vessel that ran aground in Tubattaha Reefs, Philippines, were identified as the Malayan Pangolin, *M. javanica*. They split into two groups with 3.3% mean genetic distance, suggesting at least two geographic origins.

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Pangolins; *Manis culionensis*; DNA barcoding; illegal wildlife trade; wildlife forensics

Illegal wildlife trade is one of the key threats to global biodiversity. It poses a negative impact on a species, which could lead to extinction. Illegal wildlife trade is fuelled by public demand for pets, ornaments, food, and medicine (Nijman 2010). The top 17 megadiverse countries in the world, including the Philippines, contain 75% of the global biodiversity (Heaney & Mittermeier 1997; Mittermeier et al. 1997). These megadiverse countries are targets for exploitation.

Recent reports by local and international media caught the attention of the Philippine public. For instance, the discovery of illegally traded pangolins exposed the black market to illegally traded wildlife species (Anda 2013). Asian pangolins (*Manis*, Linnaeus, 1758) are known to be exploited mainly for their meat and scales (Hsieh et al. 2011). More recent apprehensions uncovered large scale wildlife trade of exotic and endangered species such as talking mynas, blue-naped parrots, crocodiles, wild boars, and Philippine freshwater turtles (Zabanal 2015a,b; AFP 2015).

The Philippine Wildlife Resources Conservation and Protection Act (Republic Act 9147), which prohibits the capture, sale and transport of threatened species, was enacted into the law in 2001. One of the issues that hampered the implementation of the law is the ability of wildlife enforcement officers (WEOs) to correctly identify confiscated species, which is often limited to visual means. More often, tissue remains in various physical states are the only samples that can be retrieved. This poses a challenge for WEOs on how to correctly identify confiscated specimens and prosecute poachers.

DNA barcoding is a molecular technique used in wildlife forensics to rapidly and accurately identify species from samples, particularly in cases where intact specimens are not available. It uses a standard DNA region, usually the *cytochrome c oxidase I* (*COI*) for animals, as the marker of choice (Hebert et al. 2004). The COI sequence of the unknown specimens can be compared with a reference sequence available in a public database.

In this study, DNA barcoding was used to identify unknown species of confiscated pangolins from the following case reports. On 8 April 2013, a Chinese fishing vessel ran aground in Tubbataha Reef, a protected marine reserve southeast of Puerto Princesa City, Palawan, Philippines (Anda 2013). Inside the vessel were about 400 boxes containing more than 3000 frozen pangolins (AFP 2013a). In January 2014, officials of the Palawan Council for Sustainable Development Staff (PCSDS) confiscated frozen dressed pangolins in Puerto Princesa City (2014 personal communication with Atty. Adelina B. Benavente-Villena, PCSDS Legal; unreferenced). This batch was obtained from two separate residential buildings and from a vehicle (tricycle) (2014 personal communication with PCSDS personnel; unreferenced; see codes for these samples in Figure 1).

The confiscated specimens were all initially suspected to be the Palawan Pangolin (*Manis culionensis*), an endemic species distributed only in mainland Palawan and adjacent smaller islands (Lagrada et al. 2014). To verify this, COI sequences were generated from selected samples and from two live *M. culionensis* reference samples, which came from another confiscation (see AFP 2013b).

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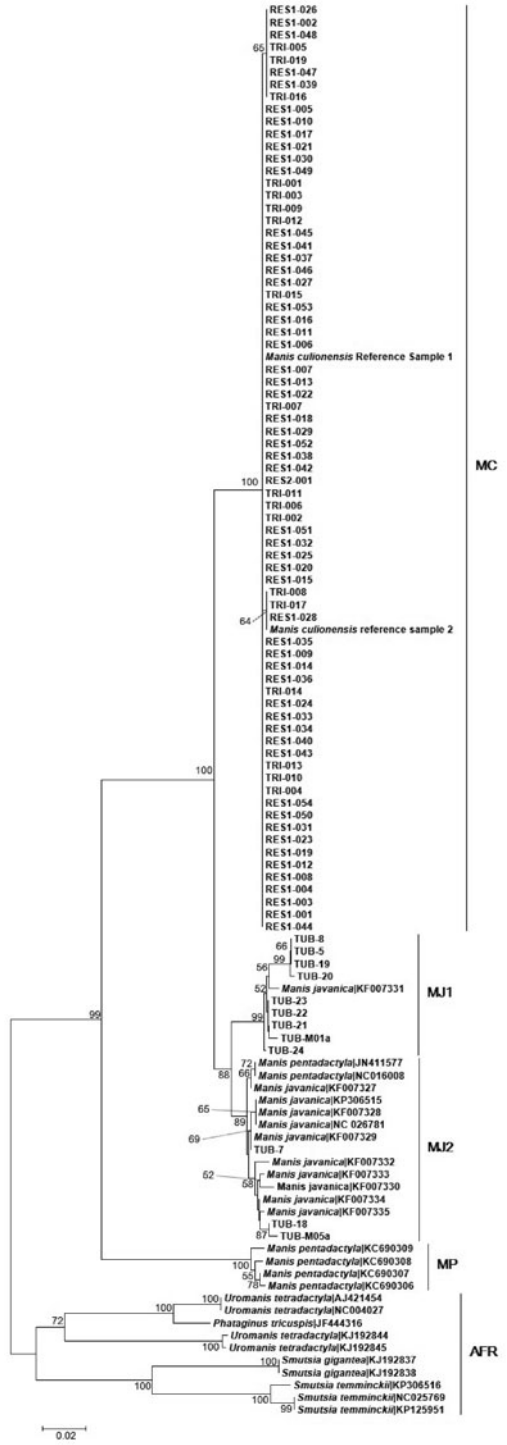

Figure 1. Unrooted COI neighbour-joining tree based on the K2P model and showing the relationships of the unknown samples, together with COI sequences of the *Manis culionensis* reference samples and other pangolin species downloaded from GenBank (taxa with accession numbers). TUB = pangolin samples acquired from the fishing vessel in Tubbataha, RES1 = pangolin samples acquired from the 1st residential area, RES2 = pangolin samples acquired from the 2nd residential area, TRI = pangolin samples acquired from a vehicle. The COI sequences split into five groups MC = *M. culionensis* group, MJ1 = *M. javanica* subgroup 1, MJ2 = *M. javanica* subgroup 2, MP = *M. pentadactyla* group, AFR = African pangolin group. Only bootstrap supports above 50% are shown in the tree. Scale bar represents two nucleotide substitutions for every 100 nucleotides.

Muscle tissues from 85 unidentified pangolins were obtained. Of the 85 specimens, 12 were from the Tubbataha case, 54 from the 1st residential building, one from the 2nd residential building, and 18 from the vehicle. The 12 specimens from Tubbataha consisted of both fresh and decomposing tissues. The rest of the muscle samples were from frozen-dressed pangolins. DNA barcodes from the two *M. culionensis* reference individuals were generated from a blood sample and a scale sample. DNA from all samples were isolated using DNeasy Blood & Tissue kit (Qiagen, USA). All tissue and genomic DNA samples are stored at the DNA Barcoding Lab, Institute of Biology, University of the Philippines, Diliman, Quezon City, Metro Manila, Philippines.

The COI region was PCR amplified using the VF1 and VR1 primers (Ivanova et al. 2006) and sent to 1st BASE Pte. Ltd. in Malaysia for DNA sequencing. Only sequences from the two *M. culionensis* reference individuals and a representative of each COI haplotype were deposited in GenBank (Accession numbers: KU207430 – KU207440, KX356690, see Table 1). COI sequences of other species of pangolins were acquired from GenBank and used as additional reference sequences (*Manis javanica*, *Manis pentadactyla*, *Phataginus tricuspis*, *Uromanis tetradactyla*, *Smutsia gigantea*, and *Smutsia temminckii*).

The dataset included a total of 111 COI sequences and was analyzed using the software MEGA 6 (Tamura et al. 2013). The sequences were first aligned using Clustal W algorithm and cut to a uniform length of 508 nucleotides. Genetic distances were corrected using the Kimura-2-parameter (K2P) and was subsequently used to construct a neighbour-joining tree (Figure 1).

In this tree, all African Pangolin species (*P. tricuspis*, *U. tetradactyla*, *S. gigantea* and *S. temminckii*) formed a distinct group from that of the Asian pangolins (*M. culionensis*, *M. pentadactyla*, and *M. tricuspis*). African vs. Asian clusters yielded an average K2P distance of 22.9% (range 20.3–26.4%). All unidentified specimens grouped with the Asian cluster.

Within the Asian pangolin cluster, four major groups were observed. *Manis culionensis* (MC) and *M. pentadactyla* (MP) each formed distinct groups (bootstrap = 100% for both), while *M. javanica* sequences split into two groups (MJ1 and MJ2; bootstrap = 99% and 89%, respectively), suggesting multiple geographic origins. Two *M. pentadactyla* GenBank sequences (JN411577 and NC016008), however, interspersed with *M. javanica* sequences. The same sequences have already been mentioned as cases of misidentification in several papers (Gaubert & Agostinho 2015; Hassanin et al. 2015).

Unidentified specimens in the first group (MC) included confiscated samples from the residential areas and the vehicle case. Only three haplotypes were observed within MC. The first haplotype was represented by *M. culionensis* reference sample 1 with 62 unidentified specimens identical to it. The second haplotype was represented by *M. culionensis* reference sample 2 with three unidentified specimens identical to it. The third haplotype contained eight sequences and only differed by one nucleotide with respect to the first two haplotypes. Sequences within MC were highly similar (average distance = 0.06%, range: 0–0.4%); therefore, the unknown samples under MC are most likely *M. culionensis*. This is also supported by preliminary morphological assessment of the

Table 1. List of COI accession numbers and samples.

GenBank accession number	Samples
KU207430	*M. culionensis* reference sample 1
	Other samples with identical sequences:
	RES1-001 RES1-003 RES1-004 RES1-005 RES1-006 RES1-007 RES1-008 RES1-009 RES1-010 RES1-011 RES1-012 RES1-013 RES1-014 RES1-015 RES1-016 RES1-017 RES1-018 RES1-019 RES1-020 RES1-021 RES1-022 RES1-023 RES1-024 RES1-025 RES1-027 RES1-029 RES1-030 RES1-031 RES1-035 RES1-032 RES1-033 RES1-034 RES1-036 RES1-037 RES1-038 RES1-040 RES1-041 RES1-042 RES1-043 RES1-044 RES1-045 RES1-046 RES1-049 RES1-050 RES1-051 RES1-052 RES1-053 RES1-054 RES2-001 TRI-001 TRI-002 TRI-003 TRI-004 TRI-006 TRI-007 TRI-009 TRI-010 TRI-011 TRI-012 TRI-013 TRI-014 TRI-015
KX356690	*M. culionensis* reference sample 2
	Other samples with an identical sequence:
	RES1-028 TRI-017 TRI-008
KU207432	TRI-016
	Other samples with an identical sequence:
	RES1-002 RES1-026 RES1-039 RES1-047 RES1-048 TRI-005 TRI-019
KU207433	TUB-7
KU207434	TUB-18
KU207435	TUB-19
	Other samples with an identical sequence:
	TUB-8 TUB-5
KU207436	TUB-20
KU207437	TUB-23
	Other samples with an identical sequence:
	TUB-22 TUB-21
KU207438	TUB-24
KU207439	TUB-M01a
KU207440	TUB-M05a

pangolin skulls. In addition, deep divergences were not observed in the group, suggesting that the samples were likely taken from a single locality. However, a population genetic study is needed to evaluate this further.

Confiscated samples from the Tubbataha case split into the two groups of *M. javanica* (MJ1 and MJ2). Nine samples grouped with one *M. javanica* sequence within MJ1, while three samples grouped with the rest of the *M. javanica* reference sequences in MJ2. There is also little genetic distance within each group (MJ1 average distance = 0.8%, range: 0.8–1.6%; MJ2 average distance = 0.8%, range: 0–1.8%) but a relatively higher distance between groups (MJ1 vs. MJ2 average distance = 3.3%, range: 2.4–4.5%). This suggests that all Tubbataha samples are most likely *M. javanica* based on the reference sequences.

Manis javanica has a wide distribution, spanning southern China to Southeast Asia (Challender et al. 2014). Although the exact locations cannot be accurately pinpointed, a deep divergence of the sequences would indicate that the Tubbataha case samples were collected from at least two regions. In addition, a 3.3% average genetic distance between MJ1 and MJ2 may indicate cryptic speciation within *M. javanica* populations. However, a more intensive population genetic study is needed to confirm this hypothesis.

Based on the COI gene tree, the closest relative of *M. culionensis* is *M. javanica*. Although the tree generated used only a single marker, it is hypothesized that the ancestor of *M. culionensis* may have migrated from Borneo to the Palawan islands. Future studies using a robust set of markers are needed to estimate divergence times.

The study demonstrates the potential of DNA barcoding in assisting WEOs in regulating wildlife trade even in the absence of intact specimens. We generated the first record of COI sequence for *M. culionensis* and compared it with COI sequences generated from unidentified pangolin specimens. All specimens were identified up to the species level, either as *M. culionensis* or *M. javanica*. The latter is not native to the Philippines, which means the poachers obtained these samples from outside the country. Many institutions have been successful in using DNA barcoding to unambiguously identify confiscated illegally traded species such as the freshwater turtle *Lissemys punctata* in Pakistan (Rehman et al. 2015), the Iguana *Conolophus subcristatus* in the Galapagos Islands (Gentile et al. 2013), and the Malayan Pangolin *M. javanica* in Hong Kong (Zhang et al. 2015). Future studies could focus on standardizing methods of DNA barcoding as a tool in wildlife forensics, which could be used as acceptable evidence during court proceedings. This is the first contribution of the Wildlife Forensics and DNA Barcoding Program of the UP Biology and Biodiversity Management Bureau of the Department of Environment and Natural Resources.

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Disclosure statement

The authors declare that there is no potential conflict of interest.

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