

DNA FORENSIC CASE STUDY: SPECIES IDENTIFICATION FROM SUSPECTED CROCODILE PENIS

***Millawati Gani¹, Noor Azleen Mohd Kulaimi^{1,2}, Norsyamimi Rosli^{1,2}, Frankie Thomas Sitam^{1,2}, Hartini Ithnin¹, Mohd Lutfi Abdullah¹ & Jeffrine J. Rovie-Ryan^{1,2}**

¹*National Wildlife Forensic Laboratory, Ex-Situ Conservation Division, Department of Wildlife and National Parks (PERHILITAN), KM 10 Jalan Cheras, 56100 Kuala Lumpur, Malaysia.*

²*Institute of Tropical Biodiversity and Sustainable Development, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia.*

*Corresponding author's email: millawati@wildlife.gov.my

ABSTRACT

Department of Wildlife and National Parks (PERHILITAN) began developing the capacity on wildlife DNA forensic since 2009 to assist in law enforcement activities. Most of the forensic cases require DNA species identification of animal parts where key morphological characters are missing. Among the cases frequently confiscated are from traditional Chinese medicine (TCM), which often claim to use animal parts such as reproductive organs. Dried crocodile penises, in particular, are believed to have medicinal benefits and are highly demanded in TCM industries since millenniums ago. In this case study, we analysed four enforcement cases comprising of 44 exhibits which resemble crocodile penis using the partial cytochrome *b* gene of the mitochondrial DNA. Sequence similarity searches were conducted using both the BLAST search engines of GenBank and also PERHILITAN's MyWILDNA database to identify the species. Out of 44 exhibits, 22 items produced DNA sequences in which three were found to be derived from *Crocodylus porosus* while the remaining was identified as *Bos taurus*, *Bos javanicus*, and *Bos indicus*. This case study showed that most of TCM which claimed to be derived from crocodile penis turned out to be counterfeit products.

Keywords: Wildlife DNA, enforcement, forensic genetics, mitochondrial DNA, cytochrome *b*

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INTRODUCTION

The application of forensic to wildlife investigation usually involves genetic species identification based on DNA sequence similarity. This field known as wildlife DNA forensics arose from conservation genetic research and forensic genetic practice to meet the emerging need for investigative tools in wildlife law enforcement and employed similar methods as human DNA forensics (Ogden *et al.*, 2009; Ahlers *et al.*, 2017; Moore & Frazier, 2019). However, species identification becomes more problematic because of most of the time only small pieces or modified body parts such as internal organs, scales, horns, ivories, teeth, skins, bones, beaks, claws, muscle fillets, and blood that are traded (Alacs *et al.*, 2010; Lyengar, 2014; Johnson, *et al.*, 2014). Understanding the importance of forensics in law enforcement, the Department of Wildlife and National Parks (PERHILITAN) began developing the capacity on wildlife DNA forensics since 2009 and have established the National Wildlife Forensic Laboratory (NWFL) complex at the PERHILITAN's headquarters in Cheras, Kuala Lumpur.

Traditional Chinese medicine (TCM) has been in existence for more than 5,000 years (Cameron *et al.*, 2004). It depends on utilising parts of wildlife (animal and plants) which form the basis of the raw ingredients for TCM. Generally, the raw ingredients may be used directly after some preparation such as grinding, washing, boiling, and drying, or may be made into factory-processed forms such as powders, pills or tablets and packaged in mass quantities (Cameron *et al.*, 2004). Almost all animal body parts can be used as TCM such as bladder, claws scale, intestine, including the reproductive organs. It is often believed that the use of wildlife reproductive organs in TCM has aphrodisiac values and therefore creating high demand for such TCMs.

Illegal wildlife trade, poaching, and smuggling is a growing problem due to the high demand for TCM, food, ornamental, pet trade, and others (Pires & Moreto, 2016). According to Tomlinson (2015), it is often believed that the more endangered and bizarre the animal is, the higher the healing effect it will have to the user. Massive exploitation of wildlife for food and traditional medicine are the major contributing factors to the illegal trade in wildlife which caused significant destruction to the ecosystems and pushed many species to the brink of extinction throughout the world. According to a survey done by Zhang *et al.* (2008) in their awareness study of wildlife trade and conservation in China, half of the respondents agreed that wildlife should be protected, but 60% of them had admitted having consumed wildlife at some point. This information indicates that wildlife awareness is there, but the law and regulation on wildlife control are insufficient.

The use of mitochondrial (mtDNA) in forensic DNA has become an important tool to assist in law enforcement agencies to curb poaching, illegal trading, and smuggling of wildlife (Lyengar, 2014). In this case study, we analysed several exhibits showing morphological similarity to crocodile penis by using the cytochrome *b* (*cob*) gene segment of the mtDNA. The central concept in DNA species identification is to match the sequence of the evidence item to a reference sequence, either through DNA sequence similarity searches (Altschul *et al.*, 1997) or by phylogenetic reconstruction.

MATERIALS AND METHODS

Four cases (Case A–D) comprising of 44 exhibit items (Figure 1) were used in this study. Genomic DNA was extracted from the exhibits using the QIAmp DNA Micro Kit (QIAGEN, Germany). Then, approximately 350 base pair (bp) of *cob* gene was PCR amplified by using the Applied Biosystems® Veriti® 96-Well Thermal Cycler (Thermo Fisher Scientific, USA). Amplification was performed using 20 µl total volume per reaction with 10 µl of GoTaq® Colorless Master Mix (Promega, USA), 2.0 µl of the universal *cob* primer pair of CYTB1/CYTB2 (Kocher *et al.*, 1989), 6 µl of nuclease-free water, and 2.0 µl of DNA template. The amplification profile for 35 cycles were pre-denaturation (95°C for 2 minutes), denaturation (95°C for 30 seconds), annealing (49°C for 30 seconds), extension (72°C for 45 seconds), post-extension (72°C for 3 minutes) and soaking (4°C). All PCR products that produced a single band were purified using illustra™ ExoProStar™ (Merck, Germany) before DNA sequencing process was performed using the Applied Biosystems® 3500 Genetic Analyzer (Thermo Fisher Scientific, USA) at NWFL.

Each DNA sequences obtained were then subjected to the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) in GenBank and also the Malaysian Wildlife DNA database (MyWILDNA) of PERHILITAN in order to validate the sequences. For the sequences identified as belonging to *Crocodylus*, genetic distances were calculated using Kimura two-parameter (Kimura, 1980) as implemented in MEGA v.7 (Kumar *et al.*, 2016). Later, a phylogenetic tree was also constructed using the neighbour-joining (NJ) method in MEGA v.7 to show the relationship of the exhibits (identified as *Crocodylus*) with two species of *Crocodylus* available in Malaysia; *Crocodylus porosus* (Saltwater crocodile) and *Crocodylus siamensis* (Siamese crocodile) by using *Crocodylus palustris* (Mugger crocodile) as outgroup.



Figure 1 Picture of exhibits used in this case study

RESULTS AND DISCUSSION

Twenty-two sequences were successfully obtained out of 44 exhibits analysed. Table 1 summarised the percentage of similarities for each sequence as compared to the GenBank and MyWILDNA databases. BLAST results revealed that 19 sequences obtained showed genetic similarities to the Family Bovidae. Three sequences (Exhibit No. Case A-01, A-02, and C-01) showed BLAST results of 100% sequences similarity to *Bos javanicus* (commonly known as Banteng) using both databases. However, *B. javanicus* is considered extinct in Peninsular Malaysia, as there are no records of sightings for more than 50 years in the wild (DWNP, 2010). Therefore, it is assumed that these exhibits are not produced locally in Peninsular Malaysia but could have been taken and produced

from other extant *B. javanicus* populations. Meanwhile, the species identification of the remaining sixteen exhibits showed that they are derived from two domestic cattle species; *Bos indicus* and *Bos taurus*, with 100% of sequence similarities. However, both species are not protected under the Wildlife Conservation Act 2010 [Act 716].

Table 1 Species identification for suspected dried crocodile penis cases

Exhibit No.	Species Verification			
	GenBank (BLASTn)		MyWILDNA database	
	Species	% similarity	Species	% similarity
Case A-01	<i>Bos javanicus</i> (Banteng)	100	<i>Bos javanicus</i> (Banteng)	100
Case A-02	<i>Bos javanicus</i> (Banteng)	100	<i>Bos javanicus</i> (Banteng)	100
Case A-04	<i>Bos taurus</i> (Domestic cattle)	100	N/A	-
Case A-05	<i>Bos taurus</i> (Domestic cattle)	100	N/A	-
Case A-07	<i>Bos taurus</i> (Domestic cattle)	100	N/A	-
Case A-10	<i>Bos taurus</i> (Domestic cattle)	100	N/A	-
Case A-14	<i>Bos taurus</i> (Domestic cattle)	100	N/A	-
Case A-07	<i>Bos taurus</i> (Domestic cattle)	100	N/A	-
Case A-18	<i>Bos taurus</i> (Domestic cattle)	100	N/A	-
Case A-19	<i>Bos taurus</i> (Domestic cattle)	100	N/A	-
Case A-22	<i>Bos taurus</i> (Domestic cattle)	100	N/A	-
Case A-23	<i>Bos taurus</i> (Domestic cattle)	100	N/A	-
Case A-27	<i>Bos taurus</i> (Domestic cattle)	100	N/A	-
Case A-28	<i>Bos taurus</i> (Domestic cattle)	100	N/A	-
Case A-32	<i>Bos taurus</i> (Domestic cattle)	100	N/A	-
Case A-34	<i>Bos taurus</i> (Domestic cattle)	100	N/A	-
Case B-01	<i>Bos indicus</i> (Domestic cattle)	100	N/A	-
Case B-02	<i>Bos indicus</i> (Domestic cattle)	100	N/A	-
Case C-01	<i>Bos javanicus</i> (Banteng)	100	<i>Bos javanicus</i> (Banteng)	100
Case D-01	<i>Crocodylus porosus</i> (Saltwater crocodile)	99.3	<i>Crocodylus porosus</i> (Saltwater crocodile)	100
Case D-02	<i>Crocodylus porosus</i> (Saltwater crocodile)	99.3	<i>Crocodylus porosus</i> (Saltwater crocodile)	100
Case D-03	<i>Crocodylus porosus</i> (Saltwater crocodile)	99.3	<i>Crocodylus porosus</i> (Saltwater crocodile)	100

N/A - there is no domestic cattle sequence in MyWILDNA database.

Only three out of 22 sequences were found to be derived from crocodile with 99.3% of sequence similarities to *C. porosus*. Pairwise distances of the three sequences as compared to crocodile species are shown in Table 2. The sequences (Exhibit No. Case D-01, D-02, and D-03) were observed to have a close genetic distance (0.6%) to *C. porosus* compared to *C. siamensis* (7.1%). According to Bradley and Baker (2001), the value of the genetic distance of <2% for *cob* indicates intraspecific variation occurring between different individuals and the higher the value, the greater the probability that two biological species may be represented. Furthermore, NJ phylogenetic tree showed that Case D-01, D-02, and D-03 were grouped with *C. porosus* with 100 bootstrap values (Clade A; Figure 2).

Table 2 Pairwise genetic distances (in percentage, %) among the ten sequences of crocodile

Exhibit No./Species	1	2	3	4	5	6	7	8	9	10
1 Case D-03										
2 Case D-01	0.0	-								
3 Case D-02	0.0	0.0	-							
4 <i>Crocodylus porosus</i> (CP20)	0.0	0.0	0.0	-						
5 <i>Crocodylus porosus</i> (JF315290)	0.6	0.6	0.0	0.6	-					
6 <i>Crocodylus porosus</i> (JF315296)	0.6	0.6	0.6	0.6	0.0	-				
7 <i>Crocodylus siamensis</i> (EF581859)	7.1	7.1	7.1	7.1	6.8	6.8	-			
8 <i>Crocodylus siamensis</i> (JF315289)	7.1	7.1	7.1	7.1	6.8	6.8	0.0	-		
9 <i>Crocodylus siamensis</i> (SIAM01)	7.1	7.1	7.1	7.1	6.8	6.8	0.0	0.0	-	
10 <i>Crocodylus palustris</i> (GU144286)	7.8	7.8	7.8	7.8	7.6	7.6	5.0	5.0	5.0	-

Overall, most of the exhibits (19 out of 22 samples) suspected to be crocodile penises are fake and are made from domestic animal's parts. Due to morphological resemblance, many consumers unknowingly purchase counterfeit products in the hopes to cure their health issues. In the black market, the price of a portion of a crocodile penis can be priced up to USD58 (~RM240) (Cruise, 2015). It is believed that crocodile penises have aphrodisiac ingredients and medicinal values, including the healing of asthma, high blood pressure, and diabetes, even to cure cancer (Tomlinson, 2015). However, there are no scientific proofs to that claim.

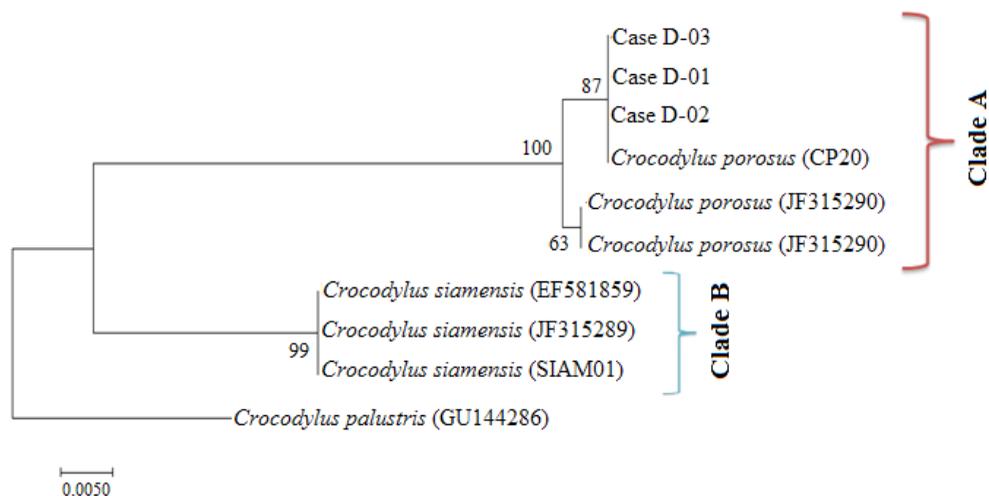


Figure 2 Neighbour-joining tree showing the relationship of the exhibits identified as crocodiles among two species of *Crocodylus* available in Malaysia by using *Crocodylus palustris* as outgroup. The number at each node represents the bootstrap value (%) based on 1000 replications

This study also showed that the *cob* gene is a useful marker for species identification even though DNA could be degraded through the manufacturing process. The *cob* gene is among the widely used gene segment in taxonomic studies and forensic science (Ogden *et al.*, 2009; Linacre, 2012). Therefore, NWFL has established the MyWILDNA database as a repository for DNA reference data for wildlife species of Malaysia using this gene segment. Ogden and Mailley (2016) have pointed out the importance of such reference data in wildlife forensic science. To enhance the MyWILDNA database as a high-quality repository for wildlife forensic applications, efforts are currently ongoing to include and deposit other gene markers (complete mitogenomes and nuclear markers). In addition, NWFL is also involved in a collaborative approach to establishing a fully-regulated database of wildlife species known as 'ForCyt' for the use in forensic investigations (Ahlers *et al.*, 2017).

PERHILITAN are facing considerable challenges to combat the illegal wildlife trade. Apart from the establishment of the NWFL to work on wildlife DNA forensics, several other measures were taken to manage this problem. This includes establishing a database and monitoring platform on wildlife trade, strengthening law enforcement to counter illegal wildlife trade for traditional medicine, drugs, food, and pet, and establishing networking with other relevant agencies both at the national and international level. More recently, PERHILITAN has made efforts to strengthen and revise the Wildlife Conservation Act 2010 as well as conducting several public awareness campaigns regarding the use of wildlife in TCM.

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