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Species Identification of The Suspected Bear Palms by The Genes of Cytb and COI

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Abstract

We reported on a case of species identification for three confiscated specimens suspected to be from bear palms. All the species of Ursidae are listed on the CITES appendices and IUCN Red List of Threatened Species. To identify the unambiguous species of confiscated animal products which are suspected from bears is very important for the purpose of wildlife conservation. In this case, cytb and COI genes of mitochondrial genome were used to identify the animal species of these confiscated specimens. The results showed that these three animal palms were identified as the species of Ursidae : one is from *Ursus thibetanus*, the others are from *Helarctos malayanus*. These two species are listed on the appendix I of CITES and classified as "Vulnerable" by the IUCN Red List of Threatened Species.

Keywords: Ursidae, animal species identification, cytb, COI

Introduction

In the present, all species of Ursidae are listed on the CITES (Convention on International Trade in Endangered Species of Wild Flora and Fauna) appendix I or II (https://www.cites.org/eng/app/appendices.php) and classified as "Vulnerable" or "Least Concern" by the IUCN (International Union for Conservation of Nature.) Red List of Threatened Species (http://www.iucnredlist.org/). Since the severe exploitation and habitat fragmentation, the populations of most species of the wild bears have been declined in the recent decades. Moreover, the activities of illegal hunting and poaching, the bears and their products still conduct for the commercial trade of pet, food (such as meat, bone, palms), or for traditional Chinese medicine of gallbladders and bile. In Taiwan, the only native bear Formosan black bear (*Ursus thibetanus formosanus*), which is the subspecies of the Asiatic black bear (*Ursus thibetanus*), is endemic in Taiwan and listed as "endangered" by Taiwan's Wildlife Conservation Act (http://conservation.forest.gov.tw/0000426) since 1989. The population distribution and habitat are restricted

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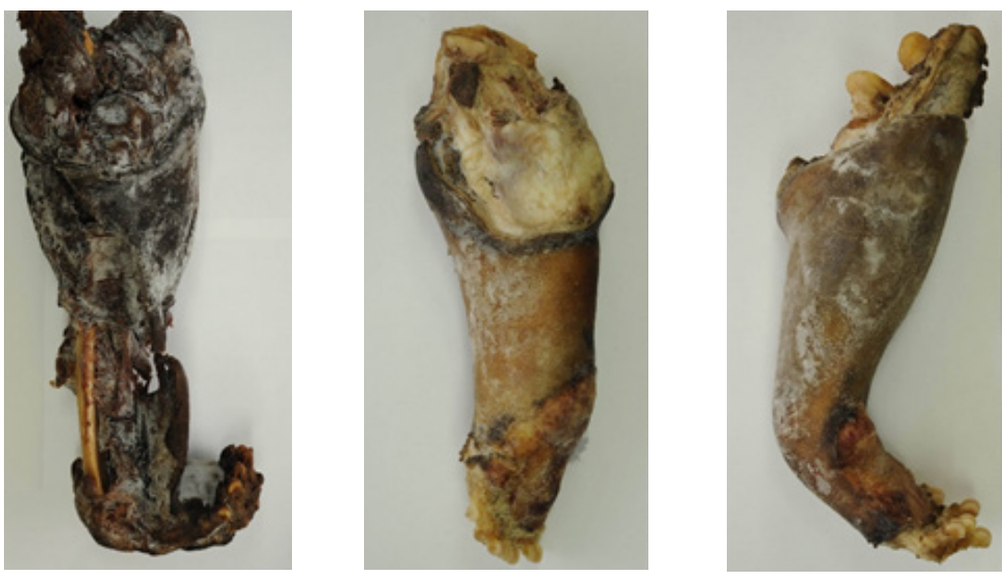
to the mountains of elevation 1,000–3,500 meters in Taiwan.

Wildlife species identifications for animal products have been conducted for more than 15 years in our laboratory. It includes various types and species, such as the suspected rhinoceros horns [1], shahtoosh furs [2], fragmented turtle shells [3-5], ivories [6], pangolin scales [7], the meat of dolphin or whale [8], avian tissues [9,10] and other animal products [11-14]. Cytochrome b (cytb) was used to identify the mammalian species in many cases and studies [1-8,11-16]. Cytochrome c oxidase subunit I (COI) has been selected as the DNA barcode for animal species identification on The Barcode of Life Data Systems (BOLD) (http://www.boldsystems.org/) and is proved to be a suitable marker for animal species identification [8,17-19]. In this case, Cytb and COI of mitochondrial genes were used on the species identification for these three suspected bear palms.

Materials and Methods

Sample source

In the summer of 2010, a banquet of bear palms was provided by a restaurant in the central-south mountain of Taiwan. Three suspected bear palms from this restaurant were confiscated by the Forestry Bureau of Council of Agriculture (COA). Our laboratory was asked to identify their species by DNA analysis. All of these animal palms have been cooked and one of them has disintegrated appearance (Fig. 1, S1). These 3 confiscated animal palms suspected to be from bears were named as S1, S2 and S3.



S1 **S2** **S3**

Fig. 1 Three confiscated specimens (S1, S2 and S3) of suspected bear palms.

DNA extraction

These specimens were washed with distilled water and cut about 100 mg of each specimen for DNA extraction. DNA extraction was performed by using Tissue & Cell Genomic DNA Purification Kit (GeneMark, Taiwan).

DNA quantification

The extracted DNA was quantified by the spectrophotometer, NanoDrop 2000 (Thermo Fisher Scientific, Delaware, USA).

PCR amplification and DNA sequencing

The cytb and COI genes were used for the species identification in this case. The primer pairs of L14724/H15915 and L14724/H15149 were designed to amplify the full length and partial length of cytb region and their PCR products were about 1344 bp and 486 bp respectively [15]. The primer pairs of COI-F1/COI-R1 were designed to amplify the full length of COI region and the PCR products were about 1779 bp. COI-F1/HCO2198 and HCO2198D-rev/COI-R1 were used to amplify the partial fragments of COI region and their PCR products were about 945 bp and 860 bp respectively. HCO2198 was designed according to the report of Folmer O et al [18], and the other primers (COI-F1, HCO2198D-rev and COI-R1) were designed by our laboratory (Table 1) [8].

Table 1 The sequences of primers for PCR amplification.

primer name[a]	sequence (5'→3')[b]
L14724	CGAAGCTTGATATGAAAAACCATCGTTG
H15149	TAACTGTAGCCCCTCAGAATGATATTTGTCCTCA
H15915	AACTGCAGTCATCTCCGGTTTACAAGAC
COI-F1	RNHYTTAGTTAACAGCTAA
HCO2198-rev	TGRTTYTTYGGNCAYCC
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA
COI-R1	RGGTTCRANTCCTTCCTTT

[a] The primers of L14724, H15915 and H15149 were designed to amplify the cytb region, and COI-F1, HCO2198D-rev, HCO2198 and COI-R1 were used to amplify the COI region.

[b] The unusual base was coded according to the IUPAC rule.

PCR amplification was performed in 50 μL of reaction mixture, which contained 50 ng extracted DNA, 1X reaction buffer, 0.2 μM dNTP, 2.5 units of Taq DNA polymerase (RICHBIO, Erlangen, Germany) and 0.2 μM each of primers. The reaction was conducted in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster, CA, USA). The PCR amplification was performed at 94℃ for 5 minutes and for 35 cycles according to the program of 94℃ for 45 seconds, 50℃ for 45 seconds and 72℃ for 1 minute, and extensions at 72℃ for 30 minutes.

PCR products were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) with both the forward and reverse primers on the thermal cycler GeneAmp PCR System 9700 (Applied Biosystems). DNA sequences of the cycle sequencing products were analyzed by the AB 3730 DNA Analyzer (Applied Biosystems).

Sequence analysis

All of the sequences excluding the primers were analyzed by the BLAST program on NCBI (National Center for Biotechnology information) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for the sequence similarity search with those registered in the GenBank and phylogenetic tree construction.

Results and Discussion

The full length of cytb and COI genes were amplified successfully from the extracted DNA of S2 and S3 specimens although they have been cooked. Only the partial cytb fragment (486 bp) and COI fragments (943 bp and 886 bp) were obtained from the S1 DNA. It was proposed that the DNA from the cooked and disintegrated specimen S1 probably was degraded seriously. After

sequencing all the PCR products mentioned above and assembling their credible sequences from forward and reverse data, the sequences were compared with the sequences registered in GenBank. Table 2 and Table 3 showed the BLAST results of sequences for cytb and COI respectively.

Table 2 The results for comparison of the cytb sequences from the three specimens with those registered in GenBank.

Specimen	Homology Species	Accession No.	Query coverage (bp/bp)	Similarity (%)
S1	*Ursus thibetanus ussuricus*	EU573176.1 AY522430.2	396/402 402/402	98.51~ 100
	Ursus thibetanus formosanus	EF076773.1	400/402	99.5
	Ursus thibetanus mupinensis	DQ402478.1	400/402	99.5
	Ursus thibetanus thibetanus	EF587265.1	399/402	99.25
	Ursus thibetanus japonicus	AB360959.1 AB863014.1	384/402 385/402	95.52~ 95.77
S2 & S3	*Helarctos malayanus*	U18899.1 FM177765.1	888/910 894/910	97.58~ 98.24
	Ursus arctos	U18875.1 U18873.1	821/909 837/908	90.32~ 92.18
	Ursus thibetanus formosanus	EF076773.1	838/910	92.09
	Ursus maritimus	KF184265.1 KF184302.1	831/909 836/908	91.42~ 92.07

Table 3 The results for comparison of the COI sequences from the three specimens with those registered in GenBank.

Specimen		Homology Species	Accession No.	Query coverage (bp/bp)	Similarity (%)
S1	F1	*Ursus thibetanus ussuricus*	EF667005.1	705/705	100
		Ursus thibetanus thibetanus	EF587265.1	701/705	99.43
		Ursus thibetanus formosanus	EF076773.1	697/705	98.86
		Ursus thibetanus mupinensis	DQ402478.1	693/705	98.30
		Ursus thibetanus japonicus	AB863014.1	682/705	96.74
	F2	*Ursus thibetanus ussuricus*	EF667005.1	810/811	99.88
		Ursus thibetanus thibetanus	EF587265.1	808/811	99.63
		Ursus thibetanus formosanus	EF076773.1	806/811	99.38
		Ursus thibetanus mupinensis	DQ402478.1	798/811	98.40
		Ursus thibetanus japonicus	AB863014.1	786/811	96.92
S2 & S3		*Helarctos malayanus*	FM177765.1 AH014080.2	1460/1477 1477/1477	98.85~ 100
		Ursus americanus	AF303109.1 KM257059.1	1368/1477 1373/1477	92.62~ 92.96
		Ursus thibetanus japonicus	AB863014.1	1364/1477	92.35
		Ursus arctos	KX641315.1 AP012589.1	1334/1477 1362/1477	90.32~ 92.21

The most similar species of S1 specimen is *Ursus thibetanus* according to the partial sequences of cytb (402 bp) and COI (F1 for 705 bp and F2 for 811 bp). The possible subspecies is *Ursus thibetanus ussuricus, Ursus thibetanus formosanus, Ursus thibetanus mupinensis* or *Ursus thibetanus thibetanus*, and their similarities with S1 are 98.30-100 %. Meanwhile the unknown specimen S1 was clustered with the species or subspecies of *Ursus thibetanus* by phylogenetic analysis except for *Ursus thibetanus japonicus* (Fig. 2). It was consistent with the BLAST results. The most similar species for both of the S2 and S3 specimens is *Helarctos malayanus* with the similarity of 97.58-98.24% and 98.85-100% for cytb (910 bp) and COI (1477 bp) respectively.

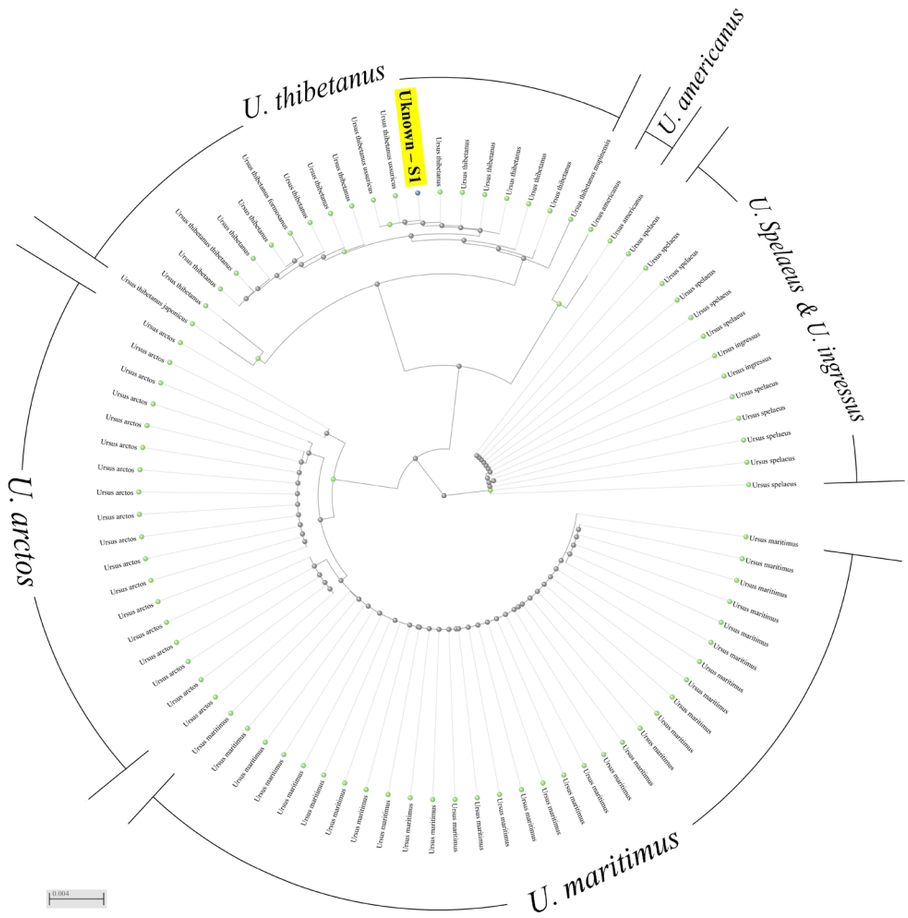


Fig. 2 The phylogenetic tree of the unknown specimen S1 (705 bp of COI) with the sequences from GenBank was constructed by the neighbor-joining method of BLAST program on NCBI website.

In this case, the DNA identification results of these three suspected bear palms showed that S1 is *Ursus thibetanus*, both S2 and S3 are *Helarctos malayanus*. These two species are listed on the appendix I of CITES and classified as “Vulnerable” by the IUCN Red List of Threatened Species. The method used in this report is valuable to identify the species of confiscated animal products for the purpose of wildlife conservation.

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