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TECHNICAL NOTE

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A DNA-Based Approach for the Forensic Identification of Asiatic Black Bear (*Ursus thibetanus*) in a Traditional Asian Medicine*

ABSTRACT: Attempts to prevent illegal trade in bile and gallbladders from Asiatic black bears, *Ursus thibetanus*, are hampered by difficulties associated with identifying such items. We extracted DNA from bile crystals of unknown species origin and generated partial cytochrome *b* (cyt *b*) sequences using either universal primers (positioned in conserved regions of cyt *b*), or primers designed on existing *U. thibetanus* sequences (UT). Species origin was determined by aligning resolved sequences to reference sequence data. The universal primers were unsuitable for *U. thibetanus* identification when multiple species templates were present in the samples. The UT primers amplified *U. thibetanus* DNA from all sample extracts, including those containing mixed species templates. The amplified fragment can distinguish *U. thibetanus* from the most closely related species, *U. americanus*, a distinct advantage of DNA sequencing over the methods currently used to analyze suspected *U. thibetanus* bile.

KEYWORDS: forensic science, species identification, *Ursus thibetanus,* traditional Chinese medicine, bear bile, gallbladder, cytochrome *b*

Bear bile has been an important constituent in traditional Asian medicine (TAM) for over 3000 years, and has been prescribed holistically for a range of ailments (1). Bile was traditionally obtained by the hunting of wild bears, primarily Asiatic black bears (*Ursus thibetanus*), for their gallbladders. More recently, declining bear populations have led to the development of farms where bile is extracted from live, often immobilized bears which are captive bred or taken from the wild (2). The bile is then sold as bile crystals or incorporated into an array of medicinal products.

U. thibetanus is classified as "vulnerable" by the World Conservation Union (3), and listed on Appendix I of the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES). International trade in the species, its parts and derivatives is therefore prohibited. However, human migration and the establishment of large expatriate Asian communities has resulted in a global market for TAM and the illegal export of bear products from Asia (2), and harvesting for the international trade is recognized as a "major threat" to the long-term survival of the species (3). Furthermore, there is evidence that bear populations outside of Asia are being targeted to supply the demand for bear gall (4). Attempts to prevent illegal international trade in animal parts and derivatives are often hampered by difficulties associated with identifying products, and are further complicated by the existence of fraudulent items. For example, purported bear gallbladders are commonly identified as domestic pig or goat by laboratory analyses (5–7).

Several techniques have been used for the species identification of suspected bear gallbladders and bile samples, including high performance liquid chromatography, thin layer chromatography (5–7), Fourier Transform Infrared analysis (7), and nuclear magnetic resonance (8). Though these techniques can distinguish genuine bear derivatives from those belonging to different species, such as pig or goat, they cannot distinguish *U. thibetanus* parts from those of other bear species. Such information is valuable within a legal framework to prove an item has crossed an international border.

Genetic techniques provide powerful tools for wildlife forensics, and can provide definitive species identifications even from unusual sample types (9,10). To our knowledge genetic techniques have not been applied to the analysis of suspected bear bile products, although they have been increasingly applied to phylogenetic and population studies within the Ursidae. As a result there is a considerable amount of published sequence data for both mitochondrial and nuclear genes (Table 1), including the mitochondrial cytochrome *b* (cyt *b*) gene that has been shown to be suitable for forensic species identification (11). Although phylogenetic relationships within the bear family remain contentious, the general consensus is that the American black bear, *U. americanus*, is the closest relative of *U. thibetanus* (12).

Here, we examine the feasibility of recovering mitochondrial DNA (mtDNA) from dried bile crystals and describe a method for the amplification and sequencing of fragments of the cyt *b* gene. We investigate two approaches: the first utilizing previously published universal primers, and the second utilizing primers we have designed using published *U. thibetanus* sequences. The resulting sequences are aligned to cyt *b* sequences in the GenBank database, and compared to reference sequences generated in-house, in order to determine the species origin of the sample.

Methods and Materials

Reference Samples

Reference samples were used for primer testing and to generate reference cyt *b* sequences. Serum samples of Asiatic black bear (*U. thibetanus)*, American black bear (*Ursus americanus)*, brown bear (*U. arctos*), polar bear (*U. maritimus*), sun bear (*Helarctos*

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TABLE 1—*Overview of DNA sequence information for nuclear and mitochondrial genes deposited in GenBank for species belonging to the subfamily Ursinae.*

Species	Nucleotide Sequences (Nuclear and Mitochondrial DNA)	Cytochrome *b* Sequences
Ursus thibetanus	61	9
Ursus americanus	340	65
Ursus arctos	789	83
Ursus maritimus	88	9
Helarctos malayanus	56	7
Melursus ursinus	31	5
Tremarctos ornatus	30	4

Totals include complete and partial sequences.

malayanus), spectacled bear (*Tremarctos ornatus*), and sloth bear (*Melursus ursinus*) were obtained together with authenticated DNA extracts of cow (*Bos taurus*), sheep (*Ovis aries*), goat (*Capra hircus*), pig (*Sus scrofa*), and human *(Homo sapiens*) (for sample sources, see Acknowledgments).

Test Samples

Two sample packages (set A and set B) each consisting of five vials of brown crystals suspected to be dried bear bile were provided for research purposes by Environment Canada (EC), the agency responsible for implementing CITES within Canada. The crystals had been confiscated by EC during CITES enforcement activities. The vials in set A were unmarked and contained large crystals. The vials in set B contained smaller crystals and were labelled with the Chinese character for "bear" (Fig. 1). The outer package also bore an image of a bear and the wording "bear bile powder" written in English.

DNA Extraction

DNA was extracted from the reference bear serum samples using an Invitrogen™ PureLink™ Genomic DNA Mini Kit following the manufacturer's protocol (Invitrogen Ltd., Paisley, U.K.). For the test samples, crystals were taken from each vial to give five replicates from each set and powdered using a Qiagen TissueLyser. DNA was extracted from 200 mg of the powder using a QIAamp Stool Mini Kit (Qiagen Inc., GmBH, Hilden, DE) following the manufacturers' protocol with the following modifications: samples were initially dissolved in 1.6 mL buffer ASL by incubation on a thermal mixer (Eppendorf AG thermomixer comfort; Eppendorf U.K. Ltd., Cambridge, U.K.) at 55°C, placed on ice for 15 min after the addition of ethanol, and washed twice in buffer AW2. DNA was eluted from the column in 50 μL of elution buffer, and total DNA was quantified by absorbance using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Labtech International, Sussex, U.K.). Extraction controls were run in parallel to all extractions.

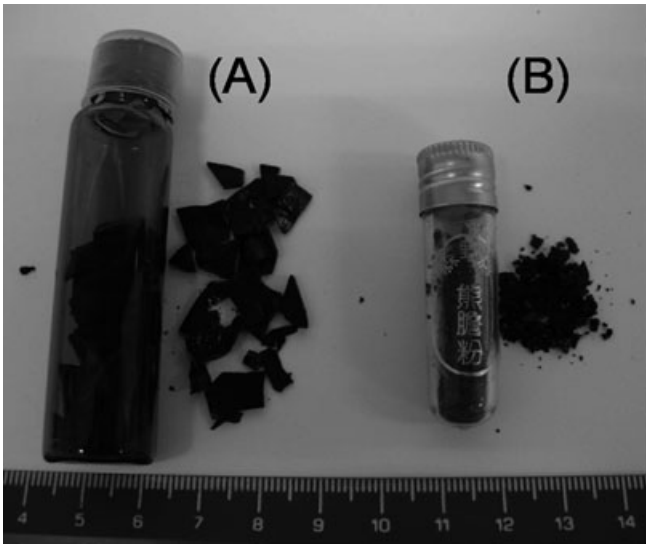

FIG. 1—*The two sets of bile crystals analyzed. Set A consisted of large crystals in unmarked vials. Set B consisted of smaller crystals in vials baring the Chinese character for "bear." Ruler indicates size in cm.*

Primer Selection

Given the unknown species origin of the test samples, two methods of amplification were investigated. The first utilized the universal primer pair mcb398 and mcb869 (13). The MCB primers amplify a region of the cyt *b* gene *c.* 420 bp in length (excluding primers) in a wide range of animal species, due to their position in a conserved region of the gene (14). The second method utilized a primer pair (UT) designed to amplify *U. thibetanus* DNA. The forward primer ut172f (GACGCGACTACAGCCTTTTC) and reverse primer ut367r (CTATGAATGCGGTGGCTATAAC) were designed using a consensus *U. thibetanus* sequence generated from seven sequences deposited in GenBank. The UT primers target a 175 bp region of the cyt *b* gene, from position 192 to 366 in the *U. thibetanus* sequence (accession number U23558). The primers were designed to mismatch the human sequence to prevent amplification of human DNA, a likely contaminant introduced during manufacture or subsequent handling. Furthermore, the primers were designed to mismatch the cyt *b* sequences of cow, goat, sheep, and pig DNA (Table 2) as bile from these species is often fraudulently sold as, or mixed with, bear bile (6).

PCR Amplification

Reaction mixtures (total volume 20 μL) contained 2 μL of template DNA, 0.36 units ABgene Thermo-Start® DNA polymerase, 1.1 mM $MgCl_2$, 0.72× reaction buffer (ABgene Ltd., Epsorn, U.K.), and 20 pmol of each primer. Amplifications were carried out using a PTC-200 MJ Research thermocycler with the following conditions. MCB Primers: initial denaturation at 95°C for 15 min, followed by 37 cycles of denaturation at 95°C for 45 sec, annealing at 50°C for 45 sec, and extension at 72°C for 1 min; followed by a final extension step at 72°C for 10 min. UT Primers: initial denaturation at 95°C for 15 min; followed by 37 cycles of: denaturation at 95°C for 30 sec, annealing at 60°C for 1 min, and extension at 72°C for 1 min; followed by a final extension step at 72°C for 5 min. All amplifications included a negative control without template DNA, and a positive control containing 5 ng of *U. thibetanus* DNA. Amplification products were visualized under UV using ethidium bromide stained agarose gels.

Sequencing of PCR Products

Amplified products were cleaned using exonuclease I and shrimp alkaline phosphatase following Werle et al. (15). Cycle sequencing of cleaned products was performed using ABI BIGDYE version 1.1 chemistries (Applied Biosystems, Foster City, CA) (to maximize sequence length of the shorter fragments) using both the forward and reverse primers, and sequencing products were resolved on an Applied Biosystems (ABI 3730xl) capillary electrophoresis

TABLE 2— *Sequences of forward and reverse UT primers, showing percentage similarity to cytochrome b sequences deposited in GenBank for the target species, U. thibetanus, and the likely adulterant or contaminant species, Sus scrofa, Bos taurus, Ovis aries, Capra hircus and Homo sapiens.*

Primer / Species																						Similarity to Primer Sequence (%)
Ut172f	5′	G	A	C	G	C	G	A	C	T	A	C	A	G	C	C	T	T	T	T	C	3′	
U. thibetanus	5′	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3′	100
S. scrofa	5′	.	.	.	A	.	A	.	.	A	.	.	.	.	.	T	.	.	C	.	.	3′	75
B. taurus	5′	.	.	.	A	.	A	.	.	A	.	.	.	.	.	A	.	.	C	.	.	3′	75
O. aries	5′	.	.	.	A	.	A	.	.	A	.	.	.	.	.	A	.	.	C	.	.	3′	75
C. hircus	5′	.	.	.	A	.	A	.	T	A	.	.	.	.	.	A	.	.	.	.	.	3′	75
H. sapiens	5′	.	.	.	.	.	C	T	.	A	.	.	C	.	.	.	.	.	.	.	.	3′	80
ut267r	5′	C	T	A	T	G	A	A	T	G	C	G	G	T	G	G	C	T	A	T	A	A	C	3′	
U. thibetanus	5′	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3′	100
S. scrofa	5′	.	.	.	.	.	.	.	G	.	.	T	.	.	T	.	.	.	.	.	.	.	.	3′	86
B. taurus	5′	.	.	.	.	A	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	.	3′	86
O. aries	5′	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	G	T	3′	82
C. hircus	5′	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	T	G	T	3′	77
H. sapiens	5′	.	.	.	.	.	.	.	G	.	.	T	.	.	T	.	.	.	.	.	.	G	T	3′	77

GenBank Acc. No.s: U. thibetanus U23558, EF076773, NC009331, AY522429, DQ402478, NC008753, AY522430; S. scrofa AJ002189; B. Taurus DQ124416; O. aries NC001941; C. hircus D84201 and H. sapiens EU073971.

instrument. Sequences were examined using CHROMAS 2.31 (Technelysium Pty, Tewantin, Qld, Australia) and a consensus sequence generated from the forward and reverse sequences using GENEIOUS version 2.5.4 software (Biomatters Ltd., Auckland, NZ). Consensus sequences were compared with sequences deposited in GenBank using a BLASTn search (16). Sequences generated from the test samples were additionally compared with those generated from the reference samples.

Results and Discussion

Extractions from both sets of crystals yielded appreciable levels of DNA (set A: 3.2–9.7 ng/μL; set B: 2.4–8.8 ng/μL), which were sufficient for subsequent PCR analysis. DNA was not detected in the extraction controls.

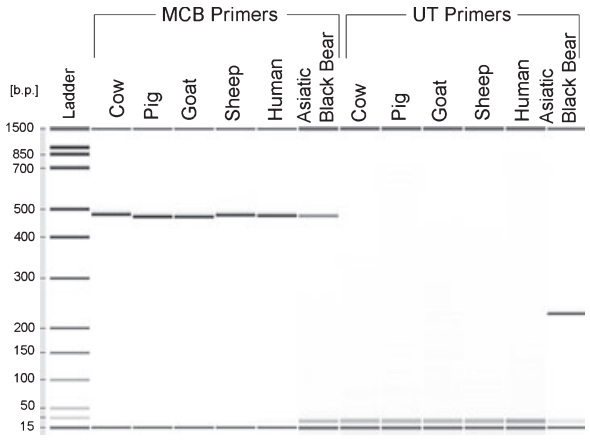

FIG. 2—*Gel image showing PCR amplification success for MCB and UT primers using authenticated reference samples of potential contaminant or adulterant species DNA. Product can clearly be seen for all species using the universal primers but not the UT primers. The UT primers are able to amplify U. thibetanus DNA (molecular weight marker supplied by Agilent Technologies UK Ltd., West Lothian, U.K.).*

Primer Testing

The MCB primers amplified DNA from all the reference samples. The UT primers amplified 175 bp fragment of DNA from each authenticated reference bear sample, with the exception of *T. ornatus.* Importantly, the UT primers did not amplify human DNA, or DNA from those species fraudulently substituted for *U. thibetanus* (Fig. 2).

Analysis of Test Samples

The universal MCB primers amplified DNA from all test sample extracts. The amplified fragment was *c.* 420 bp in length (excluding primers) of which 380 bp were compared to the sequences generated from the reference samples, and sequences deposited in GenBank (Table 3) using a BLASTn search. One of the sequences generated from set A exactly matched *H. sapiens* sequences deposited in GenBank. The remaining sequences obtained from set A were characteristic of mixed template sequencing traces, and could not be identified. All sequences generated from set B using the MCB primers were identical to each other, and most closely matched the *U. thibetanus* reference sample sequence.

The UT primers amplified 175 bp fragments from all test sample extracts. The sequences obtained from set A and set B were identical to each other and to *U. thibetanus* sequences in GenBank (Table 3). PCR products from the six authenticated reference sequences that amplified were also sequenced and matched to their respective species in GenBank. The closest match from the reference sample sequences to the test sequences was *U. thibetanus* (99.4%). The next closest species match was *U. americanus* in both GenBank (Acc. No. AF007937) and the reference sample sequences (92%). Given that molecular phylogenetic studies suggest *U. americanus* as the sister species to *U. thibetanus* (12), our data indicates the fragment generated by the UT primers can distinguish between *U. thibetanus* and its closest relative.

The quality values of the sequences analyzed indicated a less than 1% probability of error at each base position. That the sequences generated from the test samples with the MCB primers

TABLE 3—*Results of BLASTn search for fragments generated using universal (MCB) and (UT) primers, detailing most similar reference sample sequence, top species match in GenBank, GenBank accession numbers, and percentage sequence similarity.*

Sample Set/ Replicate		Primer Pair: MCB (380 bp) Reference Sample Match		MCB (380 bp) GenBank Match			UT (175 bp) Reference Sample Match		UT (175 bp) GenBank Match		
Set A	1	*H. sapiens*	99.2%	*H. sapiens*	DQ272114	100%	*U. thibetanus*	99.4%	*U. thibetanus*	AY522429	100%
	2	–	–	–	–	–	*U. thibetanus*	99.4%	*U. thibetanus*	AY522429	100%
	3	–	–	–	–	–	*U. thibetanus*	99.4%	*U. thibetanus*	AY522429	100%
	4	–	–	–	–	–	*U. thibetanus*	99.4%	*U. thibetanus*	AY522429	100%
	5	–	–	–	–	–	*U. thibetanus*	99.4%	*U. thibetanus*	AY522429	100%
Set B	1	*U. thibetanus*	98.7%	*U. thibetanus*	AY522430	99.7%	*U. thibetanus*	99.4%	*U. thibetanus*	AY522429	100%
	2	*U. thibetanus*	98.7%	*U. thibetanus*	AY522430	99.7%	*U. thibetanus*	99.4%	*U. thibetanus*	AY522429	100%
	3	*U. thibetanus*	98.7%	*U. thibetanus*	AY522430	99.7%	*U. thibetanus*	99.4%	*U. thibetanus*	AY522429	100%
	4	*U. thibetanus*	98.7%	*U. thibetanus*	AY522430	99.7%	*U. thibetanus*	99.4%	*U. thibetanus*	AY522429	100%
	5	*U. thibetanus*	98.7%	*U. thibetanus*	AY522430	99.7%	*U. thibetanus*	99.4%	*U. thibetanus*	AY522429	100%

(–) indicates mixed sequence traces which could not be identified.

TABLE 4—*Table showing polymorphisms in the sequence amplified using the UT primers, from individuals of* U. thibetanus *and* U. americanus *(sequences from GenBank and reference samples).*

Accession Number	Species	Base Position, with Reference to *U. thibetanus* Cytochrome *b* Sequence AY522429: 196	199	216	219	222	252	261	267	273	281	288	300	303	304	306	309	351	Similarity to Reference Sample (%)
Reference sample	*U. thibetanus*	G	G	C	C	T	A	C	G	C	A	A	G	C	T	G	T	C	100
AY522429	*U. thibetanus mupinensis*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100.0
AY522430	*U. thibetanus ussuricus*	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	99.4
DQ402478	*U. thibetanus mupinensis*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	99.4
EF076773	*U. thibetanus formosanus*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100.0
EF196661	*U. thibetanus*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100.0
NC008753	*U. thibetanus mupinensis*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	99.4
NC009331	*U. thibetanus formosanus*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100.0
NC009971	*U. thibetanus*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100.0
U23558	*U. thibetanus*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	99.4
Reference sample	*U. americanus*	A	A	T	T	C	T	T	A	T	G	G	–	T	C	–	C	T	92.0

were not a 100% match to the reference or published *U. thibetanus* sequences can be explained by individual variation caused by single nucleotide substitutions; such polymorphisms of the cyt *b* gene have been reported in *Ursus* species (17). The fragment amplified by the UT primers also shows some sequence variation between individuals of *U. thibetanus* (Table 4). However, a comparison of 10 sequences from the three available subspecies reveals a maximum of 1.1% intraspecific pairwise sequence divergence. Even with this level of variability the fragment is still able to distinguish *U. thibetanus* from its closest relative, *U. americanus* (minimum 8% interspecies pairwise sequence divergence).

This study demonstrates the successful recovery of *U. thibetanus* mtDNA from bile crystals, with subsequent species identification via amplification, and sequencing of a cyt *b* fragment. Although bile contains several components known to inhibit PCR (18), the DNA extraction method described here, which utilizes a commercially available extraction kit, and therefore has universal application, reduces these inhibitors to levels which permit DNA amplification.

For some replicates, *U. thibetanus* DNA could be amplified using previously published universal primers (MCB). However, a number of sequence traces generated using these primers indicated mixed template DNA. Given that human DNA was amplified from one extract (set A, replicate 1) it is likely that contamination with human DNA was the cause. As the extraction controls were clear we are confident this contamination occurred before the test samples were received into the laboratory. The UT primers, designed using previously published *U. thibetanus* sequences, allowed *U. thibetanus* DNA to be amplified from these samples. These primers are therefore more appropriate for samples containing mixed species DNA, either as a result of contamination or the adulteration of the bile with that of other known species, such as pig or goat (6).

The techniques which have been previously used to analyze suspected bear gallbladders and bear bile are able to distinguish between genuine bear parts and those belonging to other taxonomic groups, but cannot positively identify *U. thibetanus* to species level. Lin et al. (6) proposed that the species could be derived from the relative concentrations of the different bile acids detected. Seasonal changes in bile acid composition have, however, been reported in *U. americanus* (19) which may limit the accuracy of this method for species identification. The DNA sequencing method presented here has the advantage of identifying the *U. thibetanus* to the species level, verification that could be vital for confirming illegal passage of products across an international border. Furthermore, coupled with intraspecific bear phylogeographic studies (20–22), DNA sequencing may provide greater resolution of geographic origin enabling illegal international trade to be demonstrated within a species range. DNA sequencing therefore not only provides a powerful, universally available tool for the analysis of suspected bear bile samples but may assist identification of major illegal trade routes of bear products.

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References

1. Bensky D, Gamble A. Chinese herbal medicine, materia medica. Seattle, WA: Eastland Press, 1986.
2. Phillips T, Wilson P, editors. The bear bile business: the global trade in bear products from China to Asia and beyond. London: World Society for the Protection of Animals, 2002.
3. IUCN. Bear Specialist Group 1996. *Ursus thibetanus*. In: IUCN 2006 red list of threatened species, 2006. Available at: http://www.iucnredlist.org. Accessed on 6 February 2007.
4. Anderson GS. Wildlife forensic entomology: determining time of death in two illegally killed black bear cubs. J Forensic Sci 1999;44(4):856–9.
5. Espinoza EO, Shafer JA, Hagey LR. International trade in bear gall bladders—forensic source inference. J Forensic Sci 1993;38(6):1363–71.
6. Lin DL, Chang HC, Chang CP, Chen CY. Identification and differentiation of bear bile used in medicinal products in Taiwan. J Forensic Sci 1997;42(5):817–23.
7. Lin DL, Chang HC, Chen CY. Identification and quantification of bile acids in bear bile by HPLC. J Food Drug Anal 2000;8(4):283–8.
8. Theis JH, de Ropp JS, Schwab RG, Banks J, Levine K. Nuclear magnetic resonance to differentiate bear, pig and cow bile for forensic investigations. Wildlife Soc B 1988;16:430–3.
9. Lee JCI, Tsai LC, Yang CY, Liu CL, Huang LH, Linacre A, et al. DNA profiling of shahtoosh. Electrophoresis 2006;27(17):3359–62.
10. Wetton JH, Tsang CSF, Roney CA, Spriggs AC. An extremely sensitive species-specific ARMs PCR test for the presence of tiger bone DNA. Forensic Sci Int 2002;140(1):139–45.
11. Branicki W, Kupiec T, Pawlowski R. Validation of cytochrome *b* sequence analysis as a method of species identification. J Forensic Sci 2003;48(1):83–7.
12. Yu L, Li Q, Ryder OA, Zhang Y. Phylogeny of the bears (Ursidae) based on nuclear and mitochondrial genes. Mol Phy Evol 2004;32:480–94.
13. Verma SK, Singh L. Novel universal primers establish identity of an enormous number of animal species for forensic application. Mol Ecol Notes 2003;3(1):28–31.
14. Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX, et al. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc Natl Acad Sci U.S.A. 1989;86:6196–200.
15. Werle E, Schneider C, Renner M, Volke M, Fiehn W. Convenient single-step, one tube purification of PCR products for direct sequencing. Nucleic Acids Res 1994;22:4354–5.
16. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215(3):403–10.
17. Talbot SL, Shields GF. Phylogeography of Brown Bears (*Ursus arctos*) of Alaska and Paraphyly within the Ursidae. Mol Phy Evol 1996;5(3):477–9.
18. Al-Soud W, Ouis IS, Li DQ, Ljungh Å, Wadström T. Characterisation of the PCR inhibitory effect of bile to optimise real-time PCR detection of *Helicobacter* species. FEMS Immunol Med Mic 2005;44:177–82.
19. Jones JD, Zollman PE. Black bear (*Ursus americanus*) bile composition: seasonal changes. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 1997;118(3):387–90.
20. Matsuhashi T, Masuda R, Mano T, Murata K, Aiurzaniin A. Phylogenetic relationships among worldwide populations of the brown bear *Ursus arctos*. Zool Sci 2001;18(8):1137–43.
21. Marshall HD, Ritland K. Genetic diversity and differentiation of Kermode bear populations. Mol Ecol 2002;11(4):685–97.
22. Ishibashi Y, Saitoh T. Phylogenetic relationships among fragmented Asian black bear (*Ursus thibetanus*) populations in western Japan. Conser Genet 2004;5(3):311–23.

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