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NOTE

DNA barcoding reveals CITES-listed species among Taiwanese government-seized chelonian specimens

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Abstract: Compared to traditional morphological identification, DNA barcoding—molecular identification based on sequencing of a segment of mitochondrial cytochrome *c* oxidase subunit I (COI)—provides a shortcut to authenticating chelonian identifications. Here, we selected 63 government-seized chelonian specimens deposited at Taipei Zoo for DNA barcoding analysis. DNA barcoding and subsequent phylogenetic analysis successfully authenticated 36 chelonian species, including five that are listed in CITES Appendix I. Approximately 90% (57/63) of the specimens were successfully authenticated by our molecular approach, but lack or error of BOLD reference sequences, biological processes such as hybridization, and uncertain species delimitation all reduced the accuracy of DNA barcoding. To increase the accuracy of DNA barcoding, Taipei Zoo will continue to enrich the BOLD database and also establish a genetic database, to include additional genetic markers, by using government-seized chelonian specimens. A fast and accurate method to authenticate seized samples could assist law enforcement agencies to prosecute criminals and restrict illegal exploitation of wild chelonian resources.

Key words: COI, turtle, tortoise, terrapin, BOLD.

Résumé : Lorsque comparé à des méthodes traditionnelles d'identification morphologique, le codage à barres de l'ADN – une identification moléculaire fondée sur le séquençage d'un segment du gène codant pour la sous-unité I de la cytochrome *c* oxydase (COI) mitochondriale – fournit un raccourci pour l'authentification de l'identification de spécimens de chéloniens. Dans ce travail, les auteurs ont choisi 63 spécimens de chéloniens saisis par le gouvernement et déposés au Zoo de Taipei pour analyse par codage à barres. Le codage à barres et l'analyse phylogénétique qui s'en est suivie a permis d'authentifier 36 espèces de chéloniens, dont cinq qui sont sur la liste de l'annexe I du CITES. Environ 90 % (57/63) des spécimens ont été authentifiés avec succès par l'approche moléculaire, mais l'absence de séquences de référence BOLD ou des erreurs au sein de celles-ci, des processus biologiques comme l'hybridation ou encore une délimitation incertaine de l'espèce ont tous réduit la performance du codage à barres. Afin d'augmenter la justesse du codage à barres, le Zoo de Taipei va continuer à enrichir la base de données BOLD et établir une base de données génétiques, incluant des marqueurs génétiques additionnels, en exploitant les spécimens de chéloniens saisis par le gouvernement. Une méthode rapide et précise pour authentifier les échantillons saisis permettra d'aider les agences gouvernementales à poursuivre les criminels et à limiter l'exploitation illégale de chéloniens sauvages. [Traduit par la Rédaction]

Mots-clés : COI, tortue, tortue terrestre, tortue d'eau douce, BOLD.

Introduction

Chelonians (Order Testudines) have long been exploited by humans. The ancient Chinese began carving words on turtle plastrons for record-keeping and auguring at least 3000 years ago (Sung 2013), and turtle meat and plastrons are ingredients of traditional Chinese medicine (TCM). Moreover, tortoises of the genus *Geochelone* were once an important meat source for sailors before the innovation of refrigeration technology (Chambers 2006).

Modern industry has created various synthetic substitutes for turtle products, but chelonian species are still threatened. This status is due to habitat loss, invasive species, pollution, disease, climate change, and, especially, human overexploitation (Gibbons et al. 2000). Demand for chelonians as pets, as components of TCM, and for food is growing (Turtle Conservation Fund 2002; Wan et al. 2015). Based on data from the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), Luiselli et al. (2016) estimated that about 2 million wild chelonian individuals were traded during 1990–2010, with China and Hong Kong being responsible alone for more than a quarter of worldwide import trade. Apart from the legal trade, chelonians are also trafficked internationally by smuggling. In TCM markets, dry turtle shells rather than whole turtles are the main form of merchandise. Chen et al. (2009) reported that nearly 2000 metric tons of shells from hard-shelled chelonians were imported into Taiwan between 1999 and 2008. To manage chelonian resources and deter illegal trade, an easy and fast way to identify specimens to species is required; otherwise it is possible that some endangered chelonians will be overexploited given that many CITES-listed species have been found in markets (Lee et al. 2009).

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Traditional morphological and advanced DNA analyses can often be used in concert to confidently assign species identity to biological samples. Since the 5′ end (about 650 bp) of cytochrome *c* oxidase subunit I (COI, which encodes part of the terminal enzyme of the mitochondrial respiratory chain) was first employed by Hebert et al. (2003) for molecular authentication, it has been asserted that this DNA fragment could be viewed as a "DNA barcode" for biological authentication in various animals (Chang et al. 2014, 2016; Leyva-Cruz et al. 2016; Mwale et al. 2017; Ondrejicka et al. 2017). Modern DNA barcoding technology is a shortcut for species identification. Attaining the mastery of morphological taxonomy that is necessary for confident species assignment is much more difficult than conducting DNA amplification, sequencing, and analysis. It is also increasingly difficult to secure assistance from experienced taxonomists since their numbers are largely shrinking, eliciting calls for scientists to learn traditional taxonomical skills (Mora 2014; Reid et al. 2013). Here, we authenticated government-seized chelonian specimens by both morphological characters and DNA barcoding, and then assessed whether DNA barcoding could precisely authenticate chelonian species identity and thereby complement morphological identification or be an alternative to it when morphological identification is impossible. In this study, we examine the success rate of DNA barcoding for chelonian specimen identification, given the potential utility of this method for fighting the illegal trade in protected species.

Materials and methods

Sample collection

Taipei Zoo has long been the government-assigned facility for the care of seized reptiles. Customs-seized turtles were being smuggled into Taiwan, police-seized turtles were being illegally sold by retailers or illegally possessed by individuals, whereas coastguard seizures arose from apprehension of *Cuora flavomarginata* being smuggled out of Taiwan on fishing boats. Seized chelonians were identified based on morphological characters. Tissue samples (muscle, liver, or blood) were obtained from dead or ill individuals after pathological examination. Tissue samples were preserved at –20 °C, but the voucher specimens were not preserved since specimen collection and preservation is not the main work of Taipei Zoo. Overall, samples from 39 different chelonian species have been deposited in the Wildlife Cryobank of Taipei Zoo. One to three individuals for each species were selected for our study. A total of 63 individuals were barcoded (Table 1).

DNA barcoding

DNA was extracted from the 63 tissue or blood samples using a Tissue and Cell Genomic DNA Purification Kit (Genomics, Taipei, Taiwan). PCR amplifications of the partial mitochondrial COI gene (approximately 650 base pairs (bp)) were performed in a final volume reaction mixture of 50 μL containing 5 ng template DNA, 12.5 μmol of each forward and reverse primer (Ward et al. 2005; purchased from Mission Biotech Inc., Taipei, Taiwan), 25 μL of Fast-Run™ Advanced Taq Master Mix (ProTech, Taipei, Taiwan), and distilled water. The thermal cycling protocol was as follows: one cycle at 94 °C for 4 min; 35 cycles of denaturation at 94 °C for 30 s, 45–55 °C for 30 s, and 72 °C for 30 s; and, finally, a single extension step at 72 °C for 5 min. A PCR DNA Fragment Extraction Kit (Geneaid, Taipei, Taiwan) was used to purify PCR products. Approximately 50 ng of the purified PCR product was employed as template for sequencing, which was performed following the protocol of the ABI PRISM BigDye Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) with the primers used for PCR. The beginnings and ends of the contiguous sequences from both directions of the COI gene for each sample were trimmed, and then we constructed the contig sequences using the program BioEdit ver. 7.1.9 (Hall 1999). After trimming, all contig sequences started at codon position one and ended at position three, and no stop codons were found. All 63 COI sequences are submitted to GenBank, the National Centre for Biotechnology Information (NCBI; see accession numbers in Table 1). Since specimen collection and preservation is not the main work of Taipei Zoo, the vouchers from which tissue samples were obtained were not retained. The absence of the link between the sequences and their vouchers renders these barcodes unsuitable as reference sequences, so they have not been submitted to the Barcode of Life Data system (BOLD).

DNA barcoding and genetic distance-based identification

Edited COI sequences were compared to the Barcode of Life Database (BOLD) (http://www.boldsystems.org/) for specimen identification using the BOLD Identification tool (species level barcode records) and the NCBI Basic Local Alignment Search Tool (BLAST). The "Best match" and "Identity" results for each COI sequence are shown in Table 1. When we encountered an inconsistency between morphological identification and the BOLD Identification tool/NCBI BLAST results or when the BOLD Identification tool and NCBI BLAST results did not generate an unequivocal result, we performed distance-based DNA identification. COI sequences from all possible candidate species were downloaded from BOLD, as well as sequences from Chambers and Hebert (2016), Chen et al. (2013), Kehlmaier et al. (2017), Nagy et al. (2012), Parham et al. (2006), and Reid et al. (2011), to act as references. COI sequences were aligned using the TranslatorX server (http://www.translator.co.uk), which is designed to align protein-coding nucleotide sequences based on their corresponding amino acid translations (Abascal et al. 2010). A neighbor-joining (NJ) phenogram based on Kimura two-parameter (K2P) distances with 100 000 bootstrapping replicates was constructed using MEGA 7 (Kumar et al. 2016). According to the phylogenetic species concept (Nixon and Wheeler 1990), monophyly is a prerequisite for species recognition, so our specimens were authenticated based on the reference species with which they clustered and formed a monophyletic group (with high statistical support, i.e., bootstrapping value ≥ 70) in the NJ phenogram. Also, Reid et al. (2011) calculated the chelonian intraspecific K2P distance as 1.3% ± 2.2% (mean ± SD) and the mean K2P distance between species of the same genus as being 6.4%, so we only accepted authentication when the K2P distance between the query specimen and the reference species was less than 3.5% (1.3% ± 2.2%). The sequence dataset for NJ analysis is given in the supplementary data, File S1[1].

Results

For 50 of our 63 samples we obtained genetic identifications using the BOLD Identification tool or NCBI BLAST that were consistent with morphological identifications. We identified 32 species from among these 50 samples. Of these 32 species, five are listed in CITES Appendix I, 20 are listed in CITES Appendix II, 2 are listed in CITES Appendix III, and only 4 species were not CITES-listed. Based on International Union for Conservation of Nature (IUCN) categories, 4 of these 32 chelonians are Critically Endangered, 9 are Endangered, 12 are Vulnerable, and only 7 are classified as Lower Risk (Table 1). The BOLD Identification tool and NCBI BLAST both assigned sample Nos. 8 and 13 to a species that differed from the morphologically identified species. The BOLD Identification tool was also unable to assign species identity to sample Nos. 51 and 52, but the NCBI BLAST analysis revealed their sequences to be very close to that of *Chelonoidis denticulatus*. For sample Nos. 53,

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/gen-2017-0264.

Table 1. List of all samples authenticated by DNA barcoding.

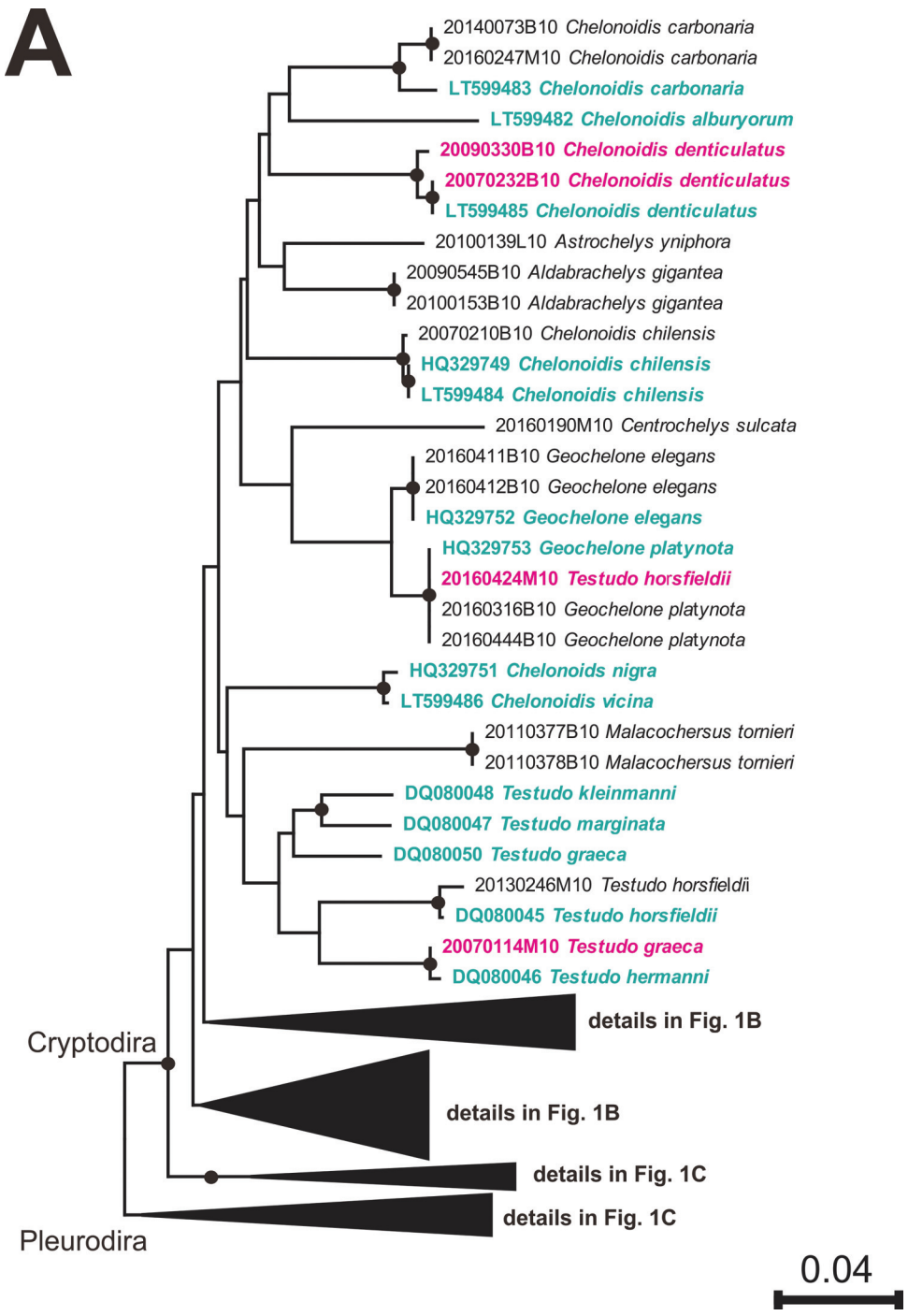
Sample No.	Date	COI sequence name/ NCBI accession No.	Morphologically identified species	BOLD database identity	NCBI BLAST	Match similarity BOLD/ Identity NCBI BLAST	CITES (2017)	IUCN (2017)	Tissue type
1	2004 Apr 28	20040137M10/MG679389	*Platysternon megacephalum*	*Platysternon megacephalum*	*Platysternon megacephalum*	100/99	I	EN	Muscle
2	2007 May 28	20070317M10/MG679390	*Platysternon megacephalum*	*Platysternon megacephalum*	*Platysternon megacephalum*	100/99	I	EN	Muscle
3	2012 Jul 6	20120276B10/MG679391	*Heosemys spinosa*	*Heosemys spinosa*	*Heosemys spinosa*	99.02/99	II	EN	Blood
4	2007 Dec 10	20070846M10/MG679392	*Mauremys japonica*	*Mauremys japonica*	*Mauremys japonica*	99.84/100	II	NT	Muscle
5	2007 Dec 17	20070862M10/MG679393	*Mauremys japonica*	*Mauremys japonica*	*Mauremys japonica*	99.84/100	II	NT	Muscle
6	2009 Mar 11	20090118B10/MG679394	*Terrapene carolina*	*Terrapene carolina*	*Terrapene carolina*	99.51/99	II	VU	Blood
7	2013 May 27	20130246M10/MG679395	*Testudo horsfieldii*	*Testudo horsfieldii*	*Testudo horsfieldii*	99.36/99	II	VU	Muscle
8	2016 Nov 21	**20160424M10/MG679396**	*Testudo horsfieldii*	*Geochelone platynota*	*Geochelone platynota*	100/100	II	VU	Muscle
9	2008 Jul 26	20080354B10/MG679397	*Cuora mouhotii*	*Cuora mouhotii*	*Cuora mouhotii*	99.06/99	II	EN	Blood
10	2010 Nov 1	20100550B10/MG679398	*Cuora mouhotii*	*Cuora mouhotii*	*Cuora mouhotii*	99.52/99	II	EN	Blood
11	2016 Nov 28	20160411B10[a]/MG679399	*Geochelone elegans*	*Geochelone elegans*	*Geochelone elegans*	100/100	II	VU	Blood
12	2016 Nov 29	20160412B10/MG679400	*Geochelone elegans*	*Geochelone elegans*	*Geochelone elegans*	100/100	II	VU	Blood
13	2007 Feb 15	**20070114M10/MG679401**	*Testudo graeca*	*Testudo hermanni*	*Testudo hermanni*	99.52/99	II	VU	Muscle
14	2010 Mar 25	20100139L10/MG679402	*Astrochelys yniphora*	*Astrochelys yniphora*	*Astrochelys yniphora*	100/100	I	CR	Liver
15	2012 Apr 13	20120128B10/MG679403	*Manouria emys*	*Manouria emys*	*Manouria emys*	100/100	II	EN	Blood
16	2012 Apr 13	20120129B10/MG679404	*Manouria emys*	*Manouria emys*	*Manouria emys*	100/100	II	EN	Blood
17	2015 Oct 1	20110647M10/MG679405	*Amyda cartilaginea*	*Amyda cartilaginea*	*Amyda cartilaginea*	98.53/99	II	VU	Muscle
18	2009 Nov 27	20090545B10/MG679406	*Aldabrachelys gigantea*	*Aldabrachelys gigantea*	*Aldabrachelys gigantea*	100/100	II	—	Blood
19	2010 Mar 30	20100153B10/MG679407	*Aldabrachelys gigantea*	*Aldabrachelys gigantea*	*Aldabrachelys gigantea*	100/100	II	—	Blood
20	2010 May 19	20100237B10/MG679408	*Mauremys reevesii*	*Mauremys reevesii*	*Mauremys reevesii*	100/100	III	EN	Blood
21	2011 Oct 24	20110584M10/MG679409	*Mauremys reevesii*	*Mauremys reevesii*	*Mauremys reevesii*	100/100	III	EN	Muscle
22	2007 Mar 27	20070210B10/MG679410	*Chelonoidis chilensis*	*Chelonoidis chilensis*	*Chelonoidis chilensis*	99.68/99	II	VU	Blood
23	2015 Mar 23	20150093B10/MG679411	*Podocnemis expansa*	*Podocnemis expansa*	*Podocnemis expansa*	100/100	II	LC	Blood
24	2012 Dec 25	20120482B10/MG679412	*Geoclemys hamiltonii*	*Geoclemys hamiltonii*	*Geoclemys hamiltonii*	99.53/100	I	VU	Blood
25	2012 Dec 26	20120483B10/MG679413	*Geoclemys hamiltonii*	*Geoclemys hamiltonii*	*Geoclemys hamiltonii*	99.53/100	I	VU	Blood
26	2010 Aug 18	20100422B10/MG679414	*Chelodina oblonga*	*Chelodina oblonga*	*Chelodina oblonga*	99.84/100	—	NT	Blood
27	2016 Feb 1	20160075M10/MG679415	*Clemmys guttata*	*Clemmys guttata*	*Clemmys guttata*	99.84/100	II	EN	Muscle
28	2014 Mar 31	20140073B10/MG679416	*Chelonoidis carbonaria*	*Chelonoidis carbonaria*	*Chelonoidis carbonaria*	97.77/98	II	—	Blood
29	2016 Jun 27	20160247M10/MG679417	*Chelonoidis carbonaria*	*Chelonoidis carbonaria*	*Chelonoidis carbonaria*	97.77/98	II	—	Muscle
30	2013 Nov 18	20130477M10/MG679418	*Cuora flavomarginata*	*Cuora flavomarginata*	*Cuora flavomarginata*	99.84/100	II	EN	Muscle
31	2015 Nov 12	20150404M10/MG679419	*Cuora flavomarginata*	*Cuora flavomarginata*	*Cuora flavomarginata*	99.84/100	II	EN	Muscle
32	2013 Dec 19	20130496M10/MG679420	*Mauremys mutica*	*Mauremys mutica*	*Mauremys mutica*	99.2/100	II	EN	Muscle
33	2015 Jul 27	20150243M10/MG679421	*Mauremys mutica*	*Mauremys mutica*	*Mauremys mutica*	99.36/99	II	EN	Muscle
34	2007 Sep 28	20070609B10/MG679422	*Cuora amboinensis*	*Cuora amboinensis*	*Cuora amboinensis*	97.72/98	II	VU	Blood
35	2008 Jul 31	20080395M10/MG679423	*Cuora amboinensis*	*Cuora amboinensis*	*Cuora amboinensis*	98.25/98	II	VU	Muscle
36	2009 Mar 27	20090136B10/MG679424	*Mauremys sinensis*	*Mauremys sinensis*	*Mauremys sinensis*	100/100	III	EN	Blood
37	2007 Apr 10	20070243B10/MG679425	*Pyxis arachnoides*	*Pyxis arachnoides*	*Pyxis arachnoides*	100/100	I	CR	Blood
38	2007 Sep 28	20070620B10/MG679426	*Pyxis arachnoides*	*Pyxis arachnoides*	*Pyxis arachnoides*	100/100	I	CR	Blood
39	2007 Jun 11	20070337M10/MG679427	*Mauremys mutica*	*Mauremys mutica*	*Mauremys mutica*	99.84/100	II	EN	Muscle
40	2010 Nov 1	20100549B10/MG679428	*Cuora galbinifrons*	*Cuora galbinifrons*	*Cuora galbinifrons*	100/100	II	CR	Blood
41	2007 Jun 11	20070338M10/MG679429	*Melanochelys trijuga*	*Melanochelys trijuga*	*Melanochelys trijuga*	99.18/98	II	NT	Muscle
42	2016 Jul 25	20160287M10/MG679430	*Chelus fimbriata*	*Chelus fimbriata*	*Chelus fimbriata*	100/100	—	—	Muscle
43	2013 Sep 12	20130432M10/MG679431	*Pelodiscus sinensis*	*Pelodiscus sinensis*	*Pelodiscus sinensis*	100/100	—	VU	Muscle
44	2011 Jun 20	20110377B10/MG679432	*Malacochersus tornieri*	*Malacochersus tornieri*	*Malacochersus tornieri*	100/99	—	VU	Blood
45	2011 Jun 20	20110378B10/MG679433	*Malacochersus tornieri*	*Malacochersus tornieri*	*Malacochersus tornieri*	100/99	—	VU	Blood
46	2016 Aug 29	20160316B10/MG679434	*Geochelone platynota*	*Geochelone platynota*	*Geochelone platynota*	100/100	I	CR	Blood
47	2016 Dec 22	20160444B10/MG679435	*Geochelone platynota*	*Geochelone platynota*	*Geochelone platynota*	99.84/99	I	CR	Blood
48	2009 Feb 9	20090053M10/MG679436	*Carettochelys insculpta*	*Carettochelys insculpta*	*Carettochelys insculpta*	99.01/99	II	VU	Muscle
49	2011 Jan 10	20110011B10/MG679437	*Carettochelys insculpta*	*Carettochelys insculpta*	*Carettochelys insculpta*	98.84/99	II	VU	Blood

Table 1 (*concluded*).

Sample No.	Date	COI sequence name/ NCBI accession No.	Morphologically identified species	BOLD database identity	NCBI BLAST	Match similarity BOLD/ Identity NCBI BLAST	CITES (2017)	IUCN (2017)	Tissue type
50	2016 Apr 25	20160190M10/MG679438	*Centrochelys sulcata*	*Centrochelys sulcata*	*Centrochelys sulcata*	100/99	II	VU	Muscle
51	2007 Apr 10	**20070232B10/MG679439**	*Chelonoidis denticulatus*	—	*Chelonoidis denticulatus*	—/100	II	VU	Blood
52	2009 Jul 14	**20090330B10/MG679440**	*Chelonoidis denticulatus*	—	*Chelonoidis denticulatus*	—/99	II	VU	Blood
53	2006 Dec 18	**20060730M10/MG679441**	*Apalone ferox*	*Apalone ferox*	*Apalone ferox*	99.84/99	III	LC	Muscle
				Pelochelys cantorii	*Pelochelys cantorii*	99.84/100			
54	2016 Jun 11	**20160230B10/MG679442**	*Stigmochelys pardalis*	*Stigmochelys pardalis*	*Stigmochelys pardalis*	98.72/99	—	LC	Blood
				Psammobates geometricus	*Psammobates geometricus*	98.25/99			
55	2016 Jun 13	**20160242M10/MG679443**	*Stigmochelys pardalis*	*Stigmochelys pardalis*	*Stigmochelys pardalis*	98.72/99	—	LC	Muscle
				Psammobates geometricus	*Psammobates geometricus*	98.25/99			
56	2009 Jul 29	**20100365M10/MG679444**	*Terrapene carolina*	*Terrapene carolina*	*Terrapene carolina*	99.84/100	II	VU	Muscle
				Terrapene mexicana	*Terrapene mexicana*	98.53/99			
57	2007 Feb 12	**20070097B10/MG679445**	*Trachemys scripta elegans*	*Trachemys scripta*	*Trachemys scripta*	100/100	—	VU	Blood
				Trachemys gaigeae	*Trachemys gaigeae*	99.02/99			
58	2008 Mar 6	**20080145M10/MG679446**	*Trachemys scripta elegans*	*Trachemys scripta*	*Trachemys scripta*	100/100	—	VU	Muscle
				Trachemys gaigeae	*Trachemys gaigeae*	99.02/99			
59	2008 Sep 11	**20080473B10/MG679447**	*Emydura subglobosa*	*Emydura subglobosa*	*Emydura subglobosa*	99.84/99	—	LC	Muscle
				Emydura tanybaraga	*Emydura tanybaraga*	99.51/99			
60	2009 Feb 2	**20090034M10/MG679448**	*Emydura subglobosa*	*Emydura subglobosa*	*Emydura subglobosa*	100/100	—	LC	Muscle
				Emydura tanybaraga	*Emydura tanybaraga*	99.67/99			
61	2015 Nov 23	**20150407M10/MG679449**	*Graptemys nigrinoda*	*Graptemys nigrinoda*	—	100/—	III	EN to LC	Muscle
				Graptemys ouachitensis	—	100/—			
				Graptemys versa	—	100/—			
				Graptemys oculifera	—	100/—			
				Graptemys pseudogeographica	*Graptemys pseudogeographica*	100/100			
				Graptemys flavimaculata	*Graptemys flavimaculata*	100/100			
				Graptemys caglei	*Graptemys caglei*	99.84/99			
				Graptemys gibbonsi	*Graptemys gibbonsi*	99.68/99			
				Graptemys barbouri	*Graptemys barbouri*	99.52/99			
				Graptemys ernsti	*Graptemys ernsti*	99.52/99			
62	2016 Aug 29	**20160346M10/MG679450**	*Graptemys nigrinoda*	*Graptemys nigrinoda*	—	100/—	III	EN to LC	Muscle
				Graptemys ouachitensis	—	100/—			
				Graptemys versa	—	100/—			
				Graptemys oculifera	—	100/—			
				Graptemys pseudogeographica	*Graptemys pseudogeographica*	100/100			
				Graptemys flavimaculata	*Graptemys flavimaculata*	100/100			
				Graptemys caglei	*Graptemys caglei*	99.84/99			
				Graptemys gibbonsi	*Graptemys gibbonsi*	99.68/99			
				Graptemys barbouri	*Graptemys barbouri*	99.52/99			
				Graptemys ernsti	*Graptemys ernsti*	99.52/99			
63	2006 May 1	**20060165M10/MG679451**	*Pseudemys peninsularis*	*Pseudemys alabamensis*	*Pseudemys alabamensis*	100/100	—	LC	Muscle
				Pseudemys rubriventris	*Pseudemys rubriventris*	99.84/99			
				—	*Pseudemys concinna concinna*	—/100			
				Pseudemys gorzugi	*Pseudemys gorzugi*	99.51/99			

Note: Sample No. and COI sequence name/NCBI accession No. in bold indicate specimens for which there was an inconsistency between the morphological identification and BOLD Identification/NCBI BLAST results or if the BOLD Identification tool and NCBI BLAST results did not generate an unequivocal result.

Fig. 1. Neighbour-joining (NJ) tree of the K2P model of 121 chelonian specimens inferred from 615 bp of mitochondrial cytochrome *c* oxidase subunit 1 (COI) sequences with 100 000 bootstrap replicates. Reference taxa are in turquoise, specimens with identical authentications based on morphological characters and both BOLD Identification and NCBI BLAST approaches are in black (see also Table 1), and specimens of questionable authentication are in magenta (see also Table 1). Scientific names of taxa in magenta and black are given based on morphological identifications. GenBank accession numbers or COI sequence identifiers are shown. Solid circles on branch nodes indicate statistically robust nodes with bootstrapping values ≥ 70 and BOLD indicates that the sequences are accessioned in the BOLD system.



54, and 55, the BOLD Identification tool and NCBI BLAST both selected two distantly related species as equally likely candidate identities. Genetic identifications could not be assigned by either BOLD or NCBI to sample Nos. 56 to 63 because these systems outputted multiple congeneric species (Table 1).

A total of 615 bp were aligned for the dataset of 121 taxa, and this combined dataset contained 280 variable sites and 269 parsimony-informative sites. Our NJ phenogram statistically supported (through bootstrapping) that sample No. 8 (20160424M10_*Testudo horsfieldii*) clusters with *Geochelone platynota* rather than any species of *Testudo*, that sample No. 13 (2070114M10_*Testudo graeca*) is phylogenetically closer to *Testudo hermanni* rather than *Testudo graeca*, and that sample Nos. 51 (20070232B10_*Chelonoidis denticulatus*) and 52 (20090330B10_*Chelonoidis denticulatus*) cluster with *Chelonoidis denticulatus* (Fig. 1A). Our NJ analysis also demonstrated that sample No. 53 (20060730M10_*Apalone ferox*) clusters among the

Fig. 1 (*continued*).

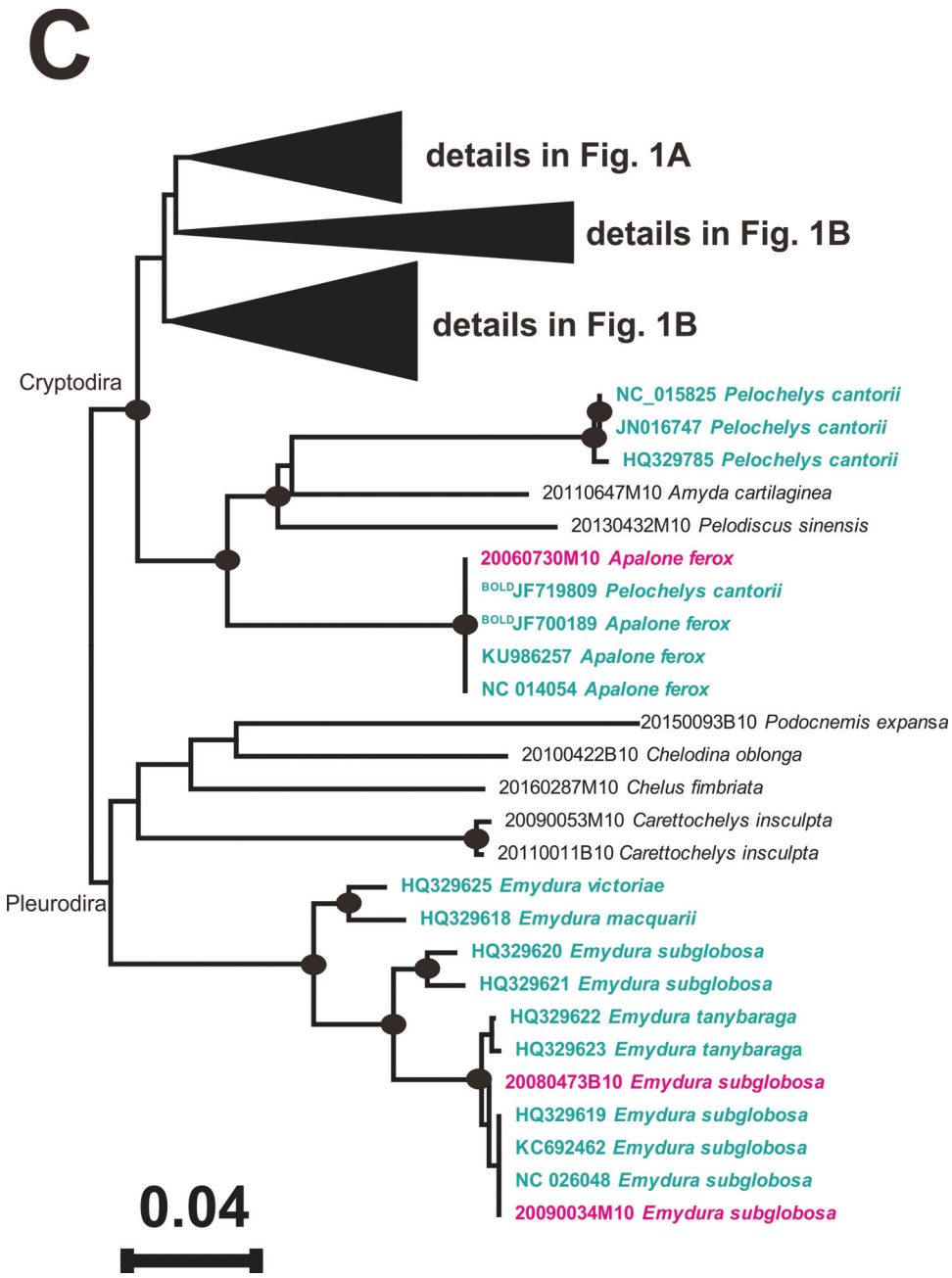
Apalone group with high statistical support (Fig. 1C), that sample Nos. 54 (20160230B10_*Stigmochelys pardalis*) and 55 (20160242M10_*Stigmochelys pardalis*) are more likely to be *Stigmochelys pardalis* rather than *Psammobates geometricus*, and that sample Nos. 57 (2007009B10_*Trachemys scripta elegans*) and 58 (20080145M10_*Trachemys scripta elegans*) are grouped with *Trachemys scripta* (Fig. 1B). However, our NJ analysis failed to clearly authenticate sample Nos. 56, and 59 to 63.

Discussion

CITES-listed species identification among confiscated chelonians

After checking the morphological identification results with both online DNA barcoding tools (BOLD and NCBI) and a distance-based clustering approach (NJ phenogram), we successfully authenticated 36 chelonian species from 57 specimens (the remaining six specimens could not be resolved, i.e., sample Nos. 56, and 59

Fig. 1 (*concluded*).



to 63). Among these 36 chelonians, 30 are CITES-listed. Since all the CITES-listed species are protected under Taiwanese law, trade of these species without government permission violates the Wildlife Conservation Act. Here, we formally confirm that endangered chelonian species are illegally traded in the Taiwanese market.

Most of these 36 chelonian species are exotic to Taiwan. Non-native reptiles are very popular pets in Taiwan (Shiau et al. 2006), so their illegal trade is largely due to market demand. However, five of these 36 chelonians, including *Mauremys reevesii*, *Mauremys sinensis*, *Mauremys mutica*, *Cuora flavomarginata*, and *Pelodiscus sinensis*, are native to Taiwan. *Mauremys reevesii*, *Mauremys mutica*, and *Cuora flavomarginata* are protected species in Taiwan. *Mauremys sinensis* is not a protected species and is very common in the pet market, whereas *Pelodiscus sinensis* is the main farmed turtle species. The government-seized specimens of these five native species were most likely being smuggled out of Taiwan to China. In recent years, a huge number of smuggled *Cuora flavomarginata* have been seized by Taiwanese authorities (Wu 2015). Turtles are an ingredient in both traditional Chinese food and medicine (Turtle Conservation Fund 2002). Since the 1980s, the economic boom in China has dramatically enhanced demand for turtles, exhausting the wild chelonian resources in China and inducing widespread legal or illegal international trade (reviewed in Gong et al. 2009). Therefore, turtle consumption in China threatens the wild chelonian resources of Taiwan, as well as other nearby countries (Chen and Lue 2010; Cheung and Dudgeon 2006).

Strengths and limitations of DNA barcoding

Under certain circumstances DNA barcoding has three advantages over traditional morphological approaches. First, DNA may be more resistant to degradation than morphological characters under some conditions. Second, a tiny amount of material (such

as muscle, blood, or shell) is sufficient for DNA extraction, so whole intact specimens are not required. Third, unlike morphological characters that vary or are absent through distinct developmental stages, resulting in species misidentification, DNA characters are constant throughout development (Chang et al. 2016). Furthermore, with rapid advances in molecular biotechnology, DNA amplification and sequencing is quick and relatively cheap. In addition, both the DNA sequencing and analysis steps are easily automated.

In this study, DNA barcoding was assessed based on sequence similarity and genetic distance. The BLAST algorithm is the basis of both the BOLD Identification tool and the NCBI system, which allows comparison between an input sequence and the reference databases based on their sequence similarity. A single species was identified by either BOLD or NCBI for more than 82% (52/63) of the specimens in this study, demonstrating that DNA barcoding is a useful tool for inspecting chelonian specimens.

Sample mislabeling can confound DNA barcoding analyses, and this is a possible reason why our DNA barcoding result for sample No. 8 (20160424M10_*Testudo horsfieldii*) was inconsistent with that of morphological identification (Table 1). However, intergeneric hybridization is not rare among chelonians (Galgon and Fritz 2002; Schild et al. 2004; Wood et al. 1983) and purposeful hybrids between *Stigmochelys pardalis* and *Centrochelys sulcata* are present in pet markets, so it cannot be ruled out that sample No. 8 is a hybrid. Amiranashvili (2000) pointed out that the morphological characters used for distinguishing *Testudo graeca* and *Testudo hermanni* are not completely reliable, so it is possible that sample No. 13 (20070114M10_*Testudo graeca*) is misidentified. Nevertheless, sample No. 13 may also be a hybrid because hybridization between *Testudo graeca* and *Testudo hermanni* has been reported (Salinas et al. 2011). Thus, both sample Nos. 8 and 13 highlight a weakness of DNA barcoding in that mitochondrial genes cannot reveal instances of hybridization, so further sequencing of nuclear genes could help to clarify the species identities of these samples.

DNA barcoding based on the partial 5′ region of the COI gene has widely been used in species identifications, but insufficient phylogenetic signal for this region preclude it from resolving phylogenetic relationships at higher taxonomic levels (Hajibabaei et al. 2006; Smith et al. 2008), perhaps explaining why the genus *Chelonoidis* is polyphyletic in our NJ phenogram (Fig. 1A). *Chelonoidis* was previously shown to be clearly monophyletic when phylogenies were based on multiple genetic markers and employing more comprehensive phylogenetic analyses (Fritz and Bininda-Emonds 2007; Kehlmaier et al. 2017).

Our NJ phenogram supported the results from both the BOLD Identification tool and NCBI BLAST that sample No. 8 is *Geochelone platynota* and that sample No. 13 is *Testudo hermanni*, and it also supported that sample Nos. 51 and 52 are *Chelonoidis denticulatus*. Moreover, it suggested that the ambiguous results for sample No. 53 very likely stem from incorrect BOLD referencing. The NJ phenogram (Fig. 1C) showed that the BOLD reference sequence of *Pelochelys cantorii* ([BOLD]JF700189_*Pelochelys cantorii*) clusters with sequences from *Apalone ferox* rather than those of *Pelochelys cantorii*, so it is probable that the BOLD reference sequence of *Pelochelys cantorii* actually comes from *Apalone ferox*. The accuracy of DNA barcoding relies on a reliable reference database. If the reference is wrong, then DNA barcoding will consequently generate an incorrect result. Both a lack of reference barcodes and outmoded or incomplete taxonomic treatments can impede the accuracy of DNA barcoding.

The ambiguous results of sample Nos. 54, 55, 57, and 58 from both the BOLD Identification tool and NCBI BLAST were resolved by our NJ phenogram. The NJ analysis supported that sample Nos. 54 and 55 were closer to *Stigmochelys pardalis* than *Psammobates geometricous*, and Nos. 57 and 58 were closer to *Trachemys scripta* than *Trachemys gaigeae*. *Stigmochelys pardalis* is the most widely distributed sub-Saharan tortoise and exhibits high intraspecific genetic diversity. A phylogenetic analysis based on multiple mitochondrial genes revealed that there are seven major clades in *Stigmochelys pardalis* (Fritz et al. 2010), so incomplete haplotype sampling from all clades may explain why our NJ analysis did not group the *Stigmochelys pardalis* specimens with high statistical support (Pollock et al. 2002) (Fig. 1B). Both our NJ phenogram and Fritz et al. (2012) supported that *Trachemys grigeae* and *Trachemys scripta* were well-supported sister species, so they could be clearly distinguished by the molecular phylogenetic analysis (Fig. 1B).

Although applied to many chelonian authentication studies (Kundu et al. 2013; Rehman et al. 2015), distance-based DNA identification cannot always accurately assign species identity. Our NJ phenogram showed that sample No. 56 was not *Terrapene carolina*, that sample Nos. 59 and 60 were either *Emydura tanybaraga* or *Emydura subglobosa*, that sample Nos. 61 and 62 were not *Graptemys nigrinoda*, and that sample No. 63 was not *Pseudemys peninsularis*, but in all these cases their actual species designation remained unclear.

The debate on taxonomy of the genus *Terrapene* is continuing (Fritz and Havaš 2014; Martin et al. 2014) because of uncertain interspecific relationships. In particular, *Terrapene carolina* has been shown not to be monophyletic by multiple phylogenetic analyses based on distinct genetic markers (Butler et al. 2010; Martin et al. 2013; Spinks et al. 2016). Moreover, Cureton et al. (2011) also evidenced gene flow between *Terrapene carolina* and *Terrapene ornata* based on microsatellite data. Until comprehensive sampling to population level is conducted and detailed genomic analyses are carried out to clarify species delimitation and relationships within *Terrapene*, we cannot identify *Terrapene* specimens based on COI sequences.

The phylogenetic analysis of Reid et al. (2011) demonstrated that *Emydura tanybaraga* and *Emydura subglobosa* are not monophyletic, which is consistent with our NJ phenogram showing that *Emydura subglobosa* is paraphyletic (some specimens grouped among *Emydura tanybaraga* sequences) (Fig. 1C). Moreover, Georges and Thomson (2010) also noticed that the morphological characters used to diagnose these two species are inadequate. DNA barcoding cannot be applied to authenticate specimens of these two species until further taxonomic work is done.

Hybridization, introgression, and incomplete lineage sorting have given rise to many taxonomically problematic chelonian groups (Shaffer et al. 2013). Wiens et al. (2010) revealed discordance between phylogenies of *Pseudemys* and *Graptemys* based on mitochondrial and nuclear markers. Spinks et al. (2013) found that *Pseudemys* suffered from taxonomic over-splitting and that no species of *Pseudemys* was monophyletic, and Thomson et al. (2018) pointed out that the taxonomy of some species of *Graptemys* needed to be revised. Consequently, our inability to assign species based on the DNA barcodes of sample Nos. 61 to 63 was not unexpected (Table 1). However, if and when *Pseudemys* and *Graptemys* are reclassified according to reliable phylogenies, molecular identification of these species based on COI and (or) other genetic makers will be feasible.

Conclusion

Overall, our study demonstrated that, in most chelonian species, DNA barcoding based on COI sequences using the online BOLD Identification tool and NCBI BLAST could easily authenticate specimen identifications and that a distance-based DNA approach improved the accuracy of DNA barcoding. Although 90% (57/63) of our samples were successfully authenticated, this study also highlights that lack or error of BOLD reference sequences, biological processes such as hybridization, and uncertain species delimitation can all erode the efficiency of DNA barcoding. To date (12 February 2018), the BOLD system houses specimen data for 261 testudinid species worldwide, of which 253 are represented by one or more DNA barcode sequences. However, on av-

erage, there are less than five barcodes for each species, and this needs to be remedied. As the principle center accommodating government-seized reptiles, Taipei Zoo can obtain many endangered species. For instance, in March 2017, Taipei Zoo received two CITES Appendix I-listed and IUCN Critically Endangered turtles, *Astrochelys yniphora* and *Batagur borneoensis*, which had been seized at an airport. Through the years, Taipei Zoo has provided appropriate environments and medical care for these seized and other captive chelonians. The staff of Taipei Zoo has amassed considerable experience with chelonians, and many endangered tortoises have successfully reproduced there, including *Astrochelys radiata*, *Geochelone platynota*, *Geochelone sulcate*, and *Indotestudo elongeta*. Moreover, the facility is responsible for public education and professional exchanges about reptiles. Taipei Zoo plans to devote more attention to chelonian genetics in the future. By utilizing its captive specimens, Taipei Zoo will not only enrich the BOLD database, but also establish a genetic database, including multiple mitochondrial and nuclear markers, to develop a more comprehensive reference database for molecular authentication of chelonian specimens, which can aid efforts to restrict the illegal exploitation of chelonian resources.

Conflict of interest statement

The authors report no conflict of interests.

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