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Short report

A molecular forensic method for identifying species composition of processed marine mammal meats

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ABSTRACT

We used universal primers designed for the cytochrome oxidase I (*CO I*) sequence of the order *Cetacea* and the family *Phocidae* to prove that meat fritters sold in Taiwan contained meat from two seal, six cetacean, and one pig species. The sequence information for *CO I* obtained in this study was limited and population genetics data for the eight sampled marine mammalian species was insufficient to deduce where these marine mammals were hunted. Regardless of the geographic origins of the marine mammal flesh, sale and consumption of marine mammals in Taiwan violates the Wildlife Conservation Act. This study provides PCR primers that could enable government testing of suspect meats to curtail the illegal trade in marine mammal products.

1. Introduction

Cetacea are loveable marine creatures that draw public attention to the issues of biodiversity and environmental conservation. Animals of the *Cetacea* order have a long history as a versatile natural resource for humans. People have used their meat, an animal source of protein, for food, and their teeth and bone as materials for traditional jewelry. Also, whale oil is still used as a fuel, an industrial lubricant, and a component of margarine. However, modern technologies mean cetacean tissue is no longer an indispensable ingredient in many products. To conserve marine biodiversity, we should cease consuming cetaceans, particularly since many cetacean creatures are endangered. Currently, the sustainably managed whale watching business, rather than the traditional whaling industry, offers the means to accomplish the goal of preserving these endangered species. There are 27 cetacean species recorded in Taiwan—all of them are protected by the government of Taiwan.[1]

Traditionally, cetacean meat has provided a protein supplement for postpartum women and has been used as an ingredient in meat fritters, giving them a special flavor; they were especially popular in Yunlin and Chiayi counties in Taiwan.[2] In 1989, the Taiwan government enacted the Wildlife Conservation Act, which protects all cetacean species in Taiwan from activities such as trading, hunting, and display. Despite this law, in January 2013, Taiwanese mass media reported that Cetacean meat fritters were being sold in Yunlin County, although the vendors claimed that they were made with seal meat imported from Canada.[3] It was difficult to verify the vendors' assertions at the time of sale because the meat fritters are marketed as homemade, as a non mass produced item. Scientific appraisal of the animal species composing the meat fritters is ongoing.

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Molecular forensics employs genetic markers to identify the species or the specific individuals represented by a sample. The technique is widely applied to traditional medicinal materials, foods and animal products in order to prevent overexploitation of protected species and to seize smuggled material.[4–10] In this study, we propose a suitable set of primers for identifying cetacean meat with a barcoding technique, thereby facilitating forensic examination of marine mammal products.

2. Materials and methods

2.1. Sample collection and DNA extraction

A total 20 meat fritters (Fig. 1) were collected from four different itinerant vendors in Yunlin County, Taiwan in 2013. We analyzed five samples from each vendor, as detailed in Table 1. Two pieces of meat were randomly selected from each meat fritter for testing. We extracted 40 DNA samples from the meat tissues using a Quick Gene DNA tissue Kit S (Fujifilm, Tokyo, Japan).

2.2. Primer design, and polymerase chain reaction (PCR)

In order to design a pair of universal primers, we downloaded 62 cytochrome c oxidase subunit I (*CO I*) haplotypes from 46 cetacean and 16 *Phocidae* species (Table 2) from GenBank and identified conserved regions with MEGA 5 software.[11] The pair of primers, SP-F (5′-CHG CHC AYG CHT TYG TRA TA-3′) and SP-R (5′-ARY ATD GTR ATN CCD GCY GC-3′) were created and tested by PCR amplification of 16 Cetacean samples obtained from the National Museum of Natural Science (NMNS), Taiwan. PCR amplifications of the partial barcode region located at the *CO I* position (366 bp) were performed with 100 ng template DNA, 12.5 μmol of each specific primer, SP-F and SP-R, 12.5 μL of Fast-Run™ Advanced *Taq* Master Mix (ProTech, Taipei, Taiwan), and distilled water in a final volume of 25 μL. Thermal cycling began with one cycle at 95 °C for 4 min, followed by 35 cycles of denaturation consisting of sequential steps of 95 °C for 0.5 min, 45 °C for 0.5 min, and 72 °C for 0.5 min, ending with a single extension step at 72 °C for 5 min. We purified the PCR products using a PCR DNA Fragment Extraction Kit (Geneaid, Taipei, Taiwan). Approximately 50 ng of the purified PCR product prepared with the SP-F primer was sequenced with an ABI PRISM BigDye Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA).

Fig. 1. Cross section of sampled meat fritters. It is suspected that the dark red meat came from marine mammals.

2.3. Data analysis

We identified the species of each amplified barcode haplotype using the Barcode of Life Database (BOLD) (http://www.boldsystems.org/) and its statistical tools.

3. Results and discussion

The universal primers, although not tested by PCR of the phocid samples, were validated by positive results when they successfully amplified the barcode region of the 16 cetacean specimens from the National Museum of Natural Science, Taiwan (Fig. 2).

The effectiveness of PCR amplification can be diminished or eliminated by food processing conditions, including physical stress, high temperature, pH, and exposure to enzymatic activity, because these may destroy the primary structure of DNA.[12,13] Fortunately, DNA extracted from the sterilized meat (121 °C for 15 min) qualified for enlarging a DNA segment to about 350 bp[14]; moreover, the accuracy of molecular identification based on the DNA barcode region sequence approaches 95% when the sequence size is 300 bp.[15] Since the length of the amplified DNA segments in this study was 366 bp, our molecular forensic identification is considered authentic. The barcode region sequences were successfully obtained from the 40 DNA specimens; among these 40 sequences, we identified 17 different haplotypes (Table 1).

Species identification with BOLD showed that the 17 haplotypes came from two seal species (*Cystophora cristata* and *Phoca groenlandica*), six cetacean species (*Grampus griseus*, *Delphinus delphis*, *Kogia breviceps*, *Steno bredanensis*, *Tursiops truncatus*, and *Feresa attenuata*), and one pig (*Sus scrofa*). The similarity values of all haplotypes evaluated by BOLD ranged from 100% to 98.62% (Table 1). The haplotypes HS1N5-2, HS1S3, HS2N3-1, HS3-1, and HS4-1 all had 100% similarity values, which fully supports that they are *C. cristata*, *P. groenlandica*, *S. scrofa*, *C. cristata*, and *Tursiops truncates*. The HS1-1, HS4N1-1, HS4N3-2, and HS4N5-1 haplotypes had 99.72% similarity values, and the HS1-2, HS2-1, and HS3N1-2 had 99.45% similarity values. The HS2-2 and HS4N4-1 both had 99.17% similarity values, and the others, HS2N2-2, HS2N4-2, and HS3N4-2, had similarity values between 99% and 98%.

The threshold for species delimitation in the barcode region is debatable, with 1%, 2%, or 3% being adopted by different researchers.[16–18] Given that the similarity values of the 16 cetacean samples from NMNS, with the exception of *Kogia sima* (NMNS12932), ranged from 100% to 98.9%, so we chose 2% as the threshold for species delimitation in this study. Since the genetic evidence suggests that the *K. sima* may contain two different species,[19] the low similarity value of the NMNS12932 may indicate that the NMNS12932 represents one *K. sima* species and the specimen recorded on the BOLD represents the other. When we used the 2% threshold for species delimitation, all haplotypes fully accomplished *molecular authentication*.

A rapid and accurate test to identify cetacean meat would fundamentally improve efforts to minimize smuggling and illegal exploitation. Compared to the immune colloidal gold strip methodology,[20] this PCR-based technique is superior because it enables species identification and is performed with extracted DNA rather than muscle tissue.[5,21,22]

The goal of identifying phocid and cetacean haplotypes was achieved by successfully amplifying the barcode segments with the designed primers. Based on the mammalian phylogeny,[23] the *Cetacea*, the *Phocidae*, and the pig all belong to the Laurasiatheria, and the pig is phylogenetically closer to the *Cetacea* than the *Phocidae* is to the *Cetacea*; hence, it is reasonable that the universal primers designed from the conserved regions of the *Cetacea* and the *Phocidae* could also be applied to the pig. Moreover, these

Table 1
Sources of meat fritters and 16 cetacean samples from the National Museum of Natural Science (NMNS), Taiwan, along with DNA barcoding results.

	Locality/museum	Date of purchase	Sample code/ voucher number	Haplotype code/ scientific name	BOLD result (similarity%)	Common name
Vendor A	Taihsi, Yunlin County	Feb. 2013	S1N1-1	HS1-2	*Phoca groenlandica* (99.45%)	Harp seal
			S1N1-2	HS1S3	*Phoca groenlandica* (100%)	Harp seal
			S1N2-1	HS1-1	*Phoca groenlandica* (99.72%)	Harp seal
			S1N2-2	HS1-1	*Phoca groenlandica* (99.72%)	Harp seal
			S1N3-1	HS1-1	*Phoca groenlandica* (99.72%)	Harp seal
			S1N3-2	HS1-1	*Phoca groenlandica* (99.72%)	Harp seal
			S1N4-1	HS1S3	*Phoca groenlandica* (100%)	Harp seal
			S1N4-2	HS1-2	*Phoca groenlandica* (99.45%)	Harp seal
			S1N5-1	HS1S3	*Phoca groenlandica* (100%)	Harp seal
			S1N5-2	HS1N5-2	*Cystophora cristata* (100%)	Hooded seal
Vendor B	Tungshih, Yunlin County	Apr. 2013	S2N1-1	HS2-1	*Grampus griseus* (99.45%)	Risso's dophin
			S2N1-2	HS2-1	*Grampus griseus* (99.45%)	Risso's dophin
			S2N2-1	HS2-2	*Delphinus delphis* (99.17%)	Common dophin
			S2N2-2	HS2N2-2	*Delphinus delphis* (98.62%)	Common dophin
			S2N3-1	HS2N3-1	*Sus scrofa* (100%)	Pig
			S2N3-2	HS2-1	*Grampus griseus* (99.45%)	Risso's dophin
			S2N4-1	HS2-2	*Delphinus delphis* (99.17%)	Common dophin
			S2N4-2	HS2N4-2	*Delphinus delphis* (98.9%)	Common dophin
			S2N5-1	HS2-2	*Delphinus delphis* (99.17%)	Common dophin
			S2N5-2	HS2-2	*Delphinus delphis* (99.17%)	Common dophin
Vendor C	Mialiao, Yunlin County	Apr. 2013	S3N1-1	HS3-1	*Cystophora cristata* (100%)	Hooded seal
			S3N1-2	HS3N1-2	*Phoca groenlandica* (99.45%)	Harp seal
			S3N2-1	HS1S3	*Phoca groenlandica* (100%)	Harp seal
			S3N2-2	HS3-1	*Cystophora cristata* (100%)	Hooded seal
			S3N3-1	HS3-1	*Cystophora cristata* (100%)	Hooded seal
			S3N3-2	HS1S3	*Phoca groenlandica* (100%)	Harp seal
			S3N4-1	HS1S3	*Phoca groenlandica* (100%)	Harp seal
			S3N4-2	HS3N4-2	*Kogia breviceps* (98.9%)	Pygmy sperm whale
			S3N5-1	HS1S3	*Phoca groenlandica* (100%)	Harp seal
			S3N5-2	HS1S3	*Phoca groenlandica* (100%)	Harp seal
Vendor D	Tungshih, Yunlin County	Apr. 2013	S4N1-1	HS4N1-1	*Steno bredanensis* (99.72%)	Rough-toothed dolphin
			S4N1-2	HS4-1	*Tursiops truncatus* (100%)	Common bottlenose dolphin
			S4N2-1	HS4-1	*Tursiops truncatus* (100%)	Common bottlenose dolphin
			S4N2-2	HS4-1	*Tursiops truncatus* (100%)	Common bottlenose dolphin
			S4N3-1	HS4-1	*Tursiops truncatus* (100%)	Common bottlenose dolphin
			S4N3-2	HS4N3-2	*Feresa attenuata* (99.72%)	Pygmy killer whale
			S4N4-1	HS4N4-1	*Steno bredanensis* (99.17%)	Rough-toothed dolphin
			S4N4-2	HS4-1	*Tursiops truncatus* (100%)	Common bottlenose dolphin
			S4N5-1	HS4N5-1	*Sus scrofa* (99.72%)	Pig
			S4N5-2	HS4-1	*Tursiops truncatus* (100%)	Common bottlenose dolphin
	NMNS		NMNS16960	*Balaenoptera acutorostrata*	*Balaenoptera acutorostrata* (99.45%)	Common minke whale
			NMNST18057	*Tursiops aduncus*	*Tursiops aduncus* (100%)	Indo-Pacific bottlenose dolphin
			TCSN-SC0901	*Sousa chinenesis*	*Sousa chinenesis* (100%)	Chinese white dolphin
			NMNS1321	*Grampus griseus*	*Grampus griseus* (100%)	Risso's dolphin
			TCSN-OO9901	*Orcinus orca*	*Orcinus orca* (100%)	Killer whale
			NMNS1320	*Steno bredanensis*	*Steno bredanensis* (99.72%)	Rough-toothed dolphin
			NMNS2375	*Stenella coeroleualba*	*Stenella coeroleualba* (99.72%)	Striped dolphin
			TCSN-PE9901	*Pepnocephala electra*	*Pepnocephala electra* (99.72%)	Melon-headed whale
			NMNS4401	*Pseudorca crassidens*	*Pseudorca crassidens* (100%)	False killer whale
			NMNS5240	*Lagenodelphis hosei*	*Lagenodelphis hosei* (99.45%)	Fraser's dolphin
			NMNS14583	*Feresa attenuate*	*Feresa attenuate* (99.72%)	Pygmy killer whale
			NMNS12932	*Kogia sima*	*Kogia sima* (91.94%)	Dwarf sperm whale
			TCSN-NP9303	*Neophocaena phocaenoides*	*Neophocaena phocaenoides* (98.9%)	Finless porpoise
			NMNS1871	*Physeter macrocephalus*	*Physeter macrocephalus* (100%)	Sperm whale
			NMNS5400	*Ziphius carvirostris*	*Ziphius carvirostris* (100%)	Cuvier's beaked whale
			TCSN-MD9602	*Mesoplodon densirostris*	*Mesoplodon densirostris* (96.97%)	Blainville's beaked whale

proposed universal primers may also make amplification of the barcode region of all the laurasiatherian organisms feasible.

To sum up, the barcoding results from the sampled meat fritters conclusively prove that both phocid and cetacean meat were sold on the Taiwanese market, thereby violating the Wildlife Conservation Act. Moreover, seals are exotic to Taiwan, and seal products cannot be legally imported into Taiwan according to Taiwan's laws and regulations; furthermore, there is no record of imported seal products in the Bureau of Foreign Trade (http://cus93.trade.gov.tw/FSCI). We consequently conjecture that the seal meat was transported into Taiwan illegally. Three out of four of the vendors sampled sold meat fritters containing cetacean flesh. The barcode data suggest that the *D. delphis* meat from Vendor B came from at least three individuals, and the *S. bredanensis* meat from Vendor D came from at least two different individuals. These six sampled cetacean species are all native to Taiwan; nevertheless, it is not possible to use genetic information in the barcode sequences to prove whether or not these six cetacean species were hunted in Taiwan. Therefore, criminal trafficking could also be contributing to the illegal cetacean meat trade.

Consumption of marine mammal flesh violates the law and harms the human body.[24–26] We have prepared a pair of universal

Table 2
The GenBank accession numbers of 46 cetacean and phocid CO I sequences used to design the pair of universal pairs.

Taxon	Scientific name	GenBank accession no.
Cetacea	*Balaenoptera borealis*	EU496284
	Balaenoptera acutorostrata	EU496285
	Balaenoptera physalus	EU496282
	Balaenoptera edeni	EU496283
	Eschrichtius robustus	EU496281
	Megaptera novaeangliae	EU496287
	Eubalaena glacialis	EU496286
	Kogia breviceps	EU496307
	Kogia sima	EU496308
	Physeter macrocephalus	EU496279
	Mesoplodon bidens	EU496312
	Mesoplodon mirus	EU496309
	Mesoplodon carlhubbsi	EU496310
	Mesoplodon europaeus	EU496313
	Mesoplodon densirostris	EU496311
	Ziphius cavirostris	EU496280
	Delphinapterus leucas	EU496288
	Pseudoorca crassidens	EU496319
	Globicephala melas	EU496303
	Globicephala macrorhynchus	EU496299
	Grampus griseus	EU496295
	Peponocephala electra	EU496291
	Feresa attenuata	EU496289
	Orcinus orca	EU496323
	Steno bredanensis	EU496375
	Sotalia fluviatilis	EU496374
	Sousa chinensis	EU496345
	Stenella frontalis	EU496350
	Stenella clymene	EU496346
	Stenella coeruleoalba	EU496341
	Stenella longirostris	EU496331
	Stenella attenuate	EU496339
	Lagenodelphis hosei	EU496355
	Tursiops aduncus	EU496330
	Tursiops truncates	EU496324
	Delphinus capensis	EU496371
	Delphinus delphis	EU496360
	Lagenorhynchus albirostris	EU496357
	Lagenorhynchus acutus	EU496356
	Pontoporia blainvillei	EU496358
	Inia geoffrensis	EU496359
	Phocoena phocoena	EU496315
	Neophocaena phocaenoides	EU496316
	Phocoena sinus	EU496314
	Phocoena spinipinnis	EU496317
	Phocoenoides dalli	EU496318
Phocidae	*Erignathus barbatus*	AY377143
	Cystophora cristata	AY377144
	Phoca vitulina	NC_001325
	Phoca largha	AY377147
	Phoca groenlandica	AY377145
	Pusa hispida	AY377146
	Pusa caspica	NC_008431
	Halichoerus grypus	NC_001602
	Monachus monachus	AY377142
	Monachus schauinslandi	AY377141
	Mirounga angustirostris	AY377138
	Mirounga leonine	AY377140
	Lobodon carcinophagus	AY377130
	Ommatophoca rossii	AY377132
	Leptonychotes weddellii	AY377136
	Hydrurga leptonyx	AY377134

primers to amplify partial barcode sequences of phocid and cetacean DNA. Our analysis of meat fritters confirmed that marine mammals were illegally captured in Taiwan and/or illegally imported. The results of this study provide primers that the government could use for testing aimed at identifying illegal trade in marine mammal flesh. We appeal to the authorities to expand efforts to stop the exploitation of these marine mammals and to enhance biodiversity conservation and protection of public health.

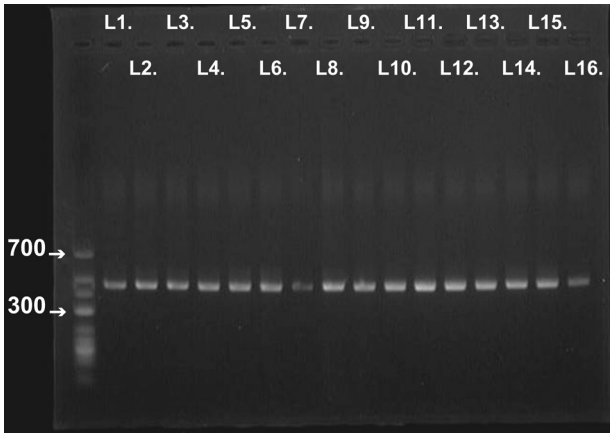

Fig. 2. Positive results of 16 cetacean species amplified by PCR with the designed primers, SP-F and SP-R. The scientific name with the specimen voucher number in parentheses of the PCR product in each loading lane is as follows. L1: *Balaenoptera acutorostrata* (NMNS16960), L2: *Tursiops aduncus* (NMNST18057), L3: *Sousa chinenesis* (TCSN-SC0901), L4: *Grampus griseus* (NMNS1321), L5: *Orcinus orca* (TCSN-OO9901), L6: *Steno bredanensis* (NMNS1320), L7: *Stenella coeroleualba* (NMNS2375), L8: *Pepnocephala electra* (TCSN-PE9901), L9: *Pseudorca crassidens* (NMNS4401), L10: *Lagenodelphis hosei* (NMNS5240), L11: *Feresa attenuate* (NMNS14583), L12: *Kogia sima* (NMNS12932), L13: *Neophocaena phocaenoides* (TCSN-NP9303), L14: *Physeter macrocephalus* (NMNS1871), L15: *Ziphius carvirostris* (NMNS5400), and L16: *Mesoplodon densirostris* (TCSN-MD9602).

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None.

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Conflict of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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