



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.

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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 bp¹⁴; 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 truncatus*. 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	<i>Phoca groenlandica</i> (99.45%)	Harp seal	
			S1N1-2	HS1S3	<i>Phoca groenlandica</i> (100%)	Harp seal	
			S1N2-1	HS1-1	<i>Phoca groenlandica</i> (99.72%)	Harp seal	
			S1N2-2	HS1-1	<i>Phoca groenlandica</i> (99.72%)	Harp seal	
			S1N3-1	HS1-1	<i>Phoca groenlandica</i> (99.72%)	Harp seal	
			S1N3-2	HS1-1	<i>Phoca groenlandica</i> (99.72%)	Harp seal	
			S1N4-1	HS1S3	<i>Phoca groenlandica</i> (100%)	Harp seal	
			S1N4-2	HS1-2	<i>Phoca groenlandica</i> (99.45%)	Harp seal	
			S1N5-1	HS1S3	<i>Phoca groenlandica</i> (100%)	Harp seal	
			S1N5-2	HS1N5-2	<i>Cystophora cristata</i> (100%)	Hooded seal	
Vendor B	Tungshih, Yunlin County	Apr. 2013	S2N1-1	HS2-1	<i>Grampus griseus</i> (99.45%)	Risso's dophin	
			S2N1-2	HS2-1	<i>Grampus griseus</i> (99.45%)	Risso's dophin	
			S2N2-1	HS2-2	<i>Delphinus delphis</i> (99.17%)	Common dophin	
			S2N2-2	HS2N2-2	<i>Delphinus delphis</i> (98.62%)	Common dophin	
			S2N3-1	HS2N3-1	<i>Sus scrofa</i> (100%)	Pig	
			S2N3-2	HS2-1	<i>Grampus griseus</i> (99.45%)	Risso's dophin	
			S2N4-1	HS2-2	<i>Delphinus delphis</i> (99.17%)	Common dophin	
			S2N4-2	HS2N4-2	<i>Delphinus delphis</i> (98.9%)	Common dophin	
			S2N5-1	HS2-2	<i>Delphinus delphis</i> (99.17%)	Common dophin	
			S2N5-2	HS2-2	<i>Delphinus delphis</i> (99.17%)	Common dophin	
			S3N1-1	HS3-1	<i>Cystophora cristata</i> (100%)	Hooded seal	
			S3N1-2	HS3N1-2	<i>Phoca groenlandica</i> (99.45%)	Harp seal	
			S3N2-1	HS1S3	<i>Phoca groenlandica</i> (100%)	Harp seal	
S3N2-2	HS3-1	<i>Cystophora cristata</i> (100%)	Hooded seal				
S3N3-1	HS3-1	<i>Cystophora cristata</i> (100%)	Hooded seal				
S3N3-2	HS1S3	<i>Phoca groenlandica</i> (100%)	Harp seal				
S3N4-1	HS1S3	<i>Phoca groenlandica</i> (100%)	Harp seal				
S3N4-2	HS3N4-2	<i>Kogia breviceps</i> (98.9%)	Pygmy sperm whale				
S3N5-1	HS1S3	<i>Phoca groenlandica</i> (100%)	Harp seal				
S3N5-2	HS1S3	<i>Phoca groenlandica</i> (100%)	Harp seal				
Vendor D	Tungshih, Yunlin County	Apr. 2013	S4N1-1	HS4N1-1	<i>Steno bredanensis</i> (99.72%)	Rough-toothed dolphin	
			S4N1-2	HS4-1	<i>Tursiops truncatus</i> (100%)	Common bottlenose dolphin	
			S4N2-1	HS4-1	<i>Tursiops truncatus</i> (100%)	Common bottlenose dolphin	
			S4N2-2	HS4-1	<i>Tursiops truncatus</i> (100%)	Common bottlenose dolphin	
			S4N3-1	HS4-1	<i>Tursiops truncatus</i> (100%)	Common bottlenose dolphin	
			S4N3-2	HS4N3-2	<i>Feresa attenuata</i> (99.72%)	Pygmy killer whale	
			S4N4-1	HS4N4-1	<i>Steno bredanensis</i> (99.17%)	Rough-toothed dolphin	
			S4N4-2	HS4-1	<i>Tursiops truncatus</i> (100%)	Common bottlenose dolphin	
			S4N5-1	HS4N5-1	<i>Sus scrofa</i> (99.72%)	Pig	
			S4N5-2	HS4-1	<i>Tursiops truncatus</i> (100%)	Common bottlenose dolphin	
			NMNS	NMNS16960	<i>Balaenoptera acutorostrata</i>	<i>Balaenoptera acutorostrata</i> (99.45%)	Common minke whale
				NMNST18057	<i>Tursiops aduncus</i>	<i>Tursiops aduncus</i> (100%)	Indo-Pacific bottlenose dolphin
				TCSN-SC0901	<i>Sousa chinensis</i>	<i>Sousa chinensis</i> (100%)	Chinese white dolphin
				NMNS1321	<i>Grampus griseus</i>	<i>Grampus griseus</i> (100%)	Risso's dolphin
		TCSN-OO9901	<i>Orcinus orca</i>	<i>Orcinus orca</i> (100%)	Killer whale		
	NMNS1320	<i>Steno bredanensis</i>	<i>Steno bredanensis</i> (99.72%)	Rough-toothed dolphin			
	NMNS2375	<i>Stenella coerulealba</i>	<i>Stenella coerulealba</i> (99.72%)	Striped dolphin			
	TCSN-PE9901	<i>Pepnocephala electra</i>	<i>Pepnocephala electra</i> (99.72%)	Melon-headed whale			
	NMNS4401	<i>Pseudorca crassidens</i>	<i>Pseudorca crassidens</i> (100%)	False killer whale			
	NMNS5240	<i>Lagenodelphis hosei</i>	<i>Lagenodelphis hosei</i> (99.45%)	Fraser's dolphin			
	NMNS14583	<i>Feresa attenuate</i>	<i>Feresa attenuate</i> (99.72%)	Pygmy killer whale			
	NMNS12932	<i>Kogia sima</i>	<i>Kogia sima</i> (91.94%)	Dwarf sperm whale			
	TCSN-NP9303	<i>Neophocaena phocaenoides</i>	<i>Neophocaena phocaenoides</i> (98.9%)	Finless porpoise			
	NMNS1871	<i>Physeter macrocephalus</i>	<i>Physeter macrocephalus</i> (100%)	Sperm whale			
	NMNS5400	<i>Ziphius cavirostris</i>	<i>Ziphius cavirostris</i> (100%)	Cuvier's beaked whale			
	TCSN-MD9602	<i>Mesoplodon densirostris</i>	<i>Mesoplodon densirostris</i> (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 primers.

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

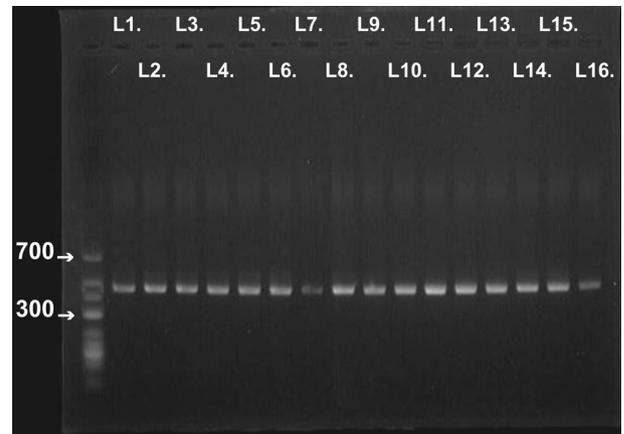


Fig. 2. Positive results of 16 cetacean species amplified by PCR with the designed primers, SP-F and SP-R. The scientific name with the specimen voucher number in parentheses of the PCR product in each loading lane is as follows. L1: *Balaenoptera acutorostrata* (NMNS16960), L2: *Tursiops aduncus* (NMNST18057), L3: *Sousa chinensis* (TCSN-SC0901), L4: *Grampus griseus* (NMNS1321), L5: *Orcinus orca* (TCSN-009901), L6: *Steno bredanensis* (NMNS1320), L7: *Stenella coeruleoalba* (NMNS2375), L8: *Peponocephala 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 cavirostris* (NMNS5400), and L16: *Mesoplodon densirostris* (TCSN-MD9602).

Ethical approval

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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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