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Genetic Differentiation between Fake Abalone and Genuine *Haliotis* Species Using the Forensically Informative Nucleotide Sequencing (FINS) Method

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ABSTRACT: Abalones (*Haliotis* species) are a popular delicacy and commonly preserved in dried form either whole or in slices or small pieces for consumption in Asian countries. Driven by the huge profit from trading abalones, dishonest traders may substitute other molluscan species for processed abalone, of which the morphological characteristics are frequently lost in the processed form. For protection of consumer rights and law enforcement against fraud, there is a need for an effective methodology to differentiate between fake and genuine abalone. This paper describes a method (validated according to the international forensic guidelines provided by SWGDAM) for the identification of fake abalone species using forensically informative nucleotide sequence (FINS) analysis. A study of the local market revealed that many claimed "abalone slice" samples on sale are not genuine. The fake abalone samples were found to be either volutids of the genus *Cymbium* (93%) or the muricid *Concholepas concholepas* (7%). This is the first report of *Cymbium* species being used for the preparation and sale as "abalone" in dried sliced form in Hong Kong.

KEYWORDS: genetic differentiation, Gastropoda, *Cymbium*, *Concholepas*, SWGDAM, Hong Kong

INTRODUCTION

Gastropods are the largest class in the phylum Mollusca. The taxonomy of Gastropoda is under constant revision as data from molecular phylogenetic analysis become available. The most recent system of gastropod classification is that of Bouchet and Rocroi.[1] The major groups of Gastropoda with economic value for food or ornamental purposes are conchs (Strombidae), whelks (Buccinoidea), abalones (Haliotidae), tops and turbans (Trochoidea), and other marine snails (mainly Neogastropoda).[2] Abalones belong to the genus *Haliotis*, the sole genus in the family Haliotidae, with a worldwide distribution in coastal temperate and tropical waters.[3] Tons of abalones are harvested commercially for food and sold at high prices in Southeast Asia. Hong Kong is one of the major cities in the international trade of seafood products such as abalones, shark fins, fish maws, and seahorses. The annual total import in Hong Kong in the 1990s was consistently >25% of the annual total world supply.[4] The high profit generated from trade in abalone has led to the selling of fake products. From the size and appearance of probable fake abalones, we judged that other large marine snails (likely to be Neogastropoda) could have been substituted. In Hong Kong, consumer rights are protected by the Trade Descriptions Ordinance (Cap. 362 of the Laws of Hong Kong). This stipulates that it is an offense for any person who in the course of any trade or business applies a false trade description to any goods or supplies or offers to supply (i.e., by exposing goods for supply) or has goods in their possession for supply to which a false trade description is applied. It is also an offense to possess for sale or for any purpose of trade or manufacture any goods to which a false trade description is applied.

Genetic identification of aquaculture products has commonly been used for law enforcement to avoid possible commercial fraud and illegal harvest of endangered species and for conservation purposes.[5−8] Genetic species identification can be done by search against homologous sequences deposited in public databases such as the NCBI GenBank or the Barcoding of Life Database (BOLD) or by using Forensically Informative Nucleotide Sequencing (FINS). FINS is a genetic species identification technique that uses phylogenetic analysis of DNA sequences.[9] It is normally performed by comparing DNA sequence variation among different species in a common gene region. Several genes within the mitochondrial genome have been widely used for inferring the relatedness of animal species such as cytochrome *b* (*cytb*), cytochrome *c* oxidase I (*COI*), and the genes that encode the 16S and 12S rRNA. Universal primers are short single strands of oligonucleotides that are able to bind to evolutionarily conserved regions of target genes across a wide diversity of animals, allowing DNA sequences to be obtained from unknown species. Genetic distances between the unknown and reference sequences are then calculated to obtain a distance matrix, and a phylogenetic tree is constructed. According to the sequence of the phylogenetic grouping of unknown samples together with reference sequences, identification can be made. Here we describe a sequence-based species identification method for differentiation of fake abalone from *Haliotis* species, validated in accordance with validation guidelines provided by the Scientific Working Group on DNA Analysis Method (SWGDAM) (http://www.cstl.nist.gov/div831/strbase/validation/SWGDAM_Validation.doc).[10,11] The validation examines the applicability or

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Table 1. Reference Materials or Reference DNA Sequences Included in This Work

					NCBI accession no.	
clade	family/superfamily	scientific name	common name[a]	sample size	16S rRNA	COI
Neogastropoda	Volutidae/ Muricoidea	*Cymbium cymbium*[b]	false elephant's snout	3	HQ260579–HQ260581	
		Cymbium pepo[b]	Neptune volute	3	HQ260582–HQ260584	JF728877–JF728879
		Cymbium tritonis[b]		3	HQ260585–HQ260587	JF728880–JF728882
		Melo melo[b]	baler shell	3	HQ260588–HQ260590	JF728883–JF728885
	Melongenidae/ Buccinoidea	*Hemifusus colosseus*[b]		3	HQ260591–HQ260593	JF728886–JF728888
	Muricidae/ Muricoidea	*Concholepas concholepas* (Bruguière, 1789) [BMNH reg. no. 19990303][c]	false abalone/loco/barnacle rock-shell		FN677453	EU391581
		Concholepas concholepas (frozen meat)[b]		3	HQ260594–HQ260596	JF728889–JF728891
			dried conch slice from Chile	1		
			dried conch slice from America	1		
			dried conch slice from West Africa	1		
			dried conch slice from Senegal	1		
	Conidae/Conoidea	*Conus textile*[d]			NC_008797	
	Turridae/Conoidea	*Lophiotoma cerithiformis*[d]			NC_008098	
	Muricidae/Muricoidea	*Rapana venosa*[d]			NC_011193	
		Thais clavigera[d]			NC_010090	
	Volutidae/Muricoidea	*Cymbium olla*[d]			NC_013245	
	Nassariidae/ Buccinoidea	*Ilyanassa obsolete*[d]			NC_007781	
	Cancellariidae/ Cancellarioidea	*Cancellaria cancellata*[d]			NC_013241	
Vetigastropoda	Haliotidae/ Haliotoidea		dried abalone from China	1		
			dried abalone from Japan	1		
		Haliotis asinina[d]	donkey's ear abalone		AY650173	
		Haliotis corrugata[d]	pink abalone		AY650172	
		Haliotis discus[d]	disk abalone		AY650174	
		Haliotis diversicolor[d]	varicolored abalone		AY650171	
		Haliotis fulgens[d]	southern green abalone		AY650158	
		Haliotis gigantea[d]	giant abalone		AY650160	
		Haliotis iris[d]	Paua abalone		AY650166	
		Haliotis midae[d]	perlemoen abalone		AY650167	
		Haliotis ovina[d]	oval abalone		AY650154	
		Haliotis rubra[d]	blacklip abalone		NC_005940	
		Haliotis rufescens[d]	red abalone		AY650164	
		Haliotis tuberculata[d]	European ormer		AY650168	
		Haliotis australis[d]			AY650157	
		Haliotis cracherodii[d]			AY650159	
		Haliotis cyclobates[d]			AY650153	
		Haliotis discus hannai[d]			EU636208	

Table 1. Continued

clade	family/superfamily	scientific name	common name[a]	sample size	NCBI accession no. 16S rRNA	NCBI accession no. COI
		Haliotis dohrniana[d]			AY650152	
		Haliotis glabra[d]			AY650151	
		Haliotis kamtschatkana[d]			AY650163	
		Haliotis laevigata[d]			AY650169	
		Haliotis pourtalesii[d]			AY650165	
		Haliotis pustulata[d]			AY650175	
		Haliotis roberti[d]			AY650150	
		Haliotis roei[d]			AY650170	
		Haliotis rugosa[d]			AY650176	
		Haliotis scalaris[d]			AY650156	
		Haliotis sorenseni[d]			AY650161	
		Haliotis varia[d]			AY650149	
		Haliotis walallensis[d]			AY650162	
		Haliotis virginea[d]			DQ276991	
Pulmonata	Clausiliidae/ Clausilioidea	*Albinaria coerulea*[d] (outgroup)			NC_001761	

[a] The common names of species used here are the official name according to the U.S. FDA, Canadian Food Inspection Agency (CFIA), or Encyclopedia of Life Support System (EOLSS). [b] DNA sequences generated in the present study. [c] DNA sequences was provided by Martine Claremont, Natural History Museum.[28] [d] DNA sequences retrieved from public database GenBank for FINS analysis.

suitability of the method developed and its sensitivity, reproducibility, stability, and optimization of the PCR conditions. A small-scale market survey was conducted using the developed method with the objective of getting an idea of the current market situation with respect to the authenticity of abalone and abalone products on sale in the local market.

MATERIALS AND METHODS

Sample Collection. A total of five species (three individuals of each) of Neogastropoda were freshly collected from different locations around the world (Table 1) and used for building the reference DNA sequences. Once received, each of the samples was given a unique label and kept frozen at −20 °C. Identification was based on the external morphological characteristics, using standard reference works and the collections of the Nature History Museum, London (NHM). Dried seafood reference materials including two whole dried abalones originating in China and Japan and four dried sliced gastropods from Chile, Senegal, the United States, and West Africa, respectively, were used for the development of the analytical protocol. Marine Products Association, an association promoting wildlife conservation and sustainable use of wildlife resources through public education, was responsible for sourcing the above fresh and dried seafood reference materials through a commission from Hong Kong Customs and Excise Department (HKCE).

DNA Extraction. Genomic DNA was extracted from a representative 25−50 mg portion of the muscle tissue of the respective sample, using a QIAGEN DNeasy Tissue Kit. The extraction procedure was based on the manufacturer's recommended protocol. Polyethylene glycol (PEG6000, USB) was added to AL buffer at a final concentration of 1% to enhance removal of mucopolysaccharides. A negative control was included in the extraction process in each batch of analysis to check for contamination. The DNA content and quality of the extracts were evaluated by UV spectrometric analysis.

Amplification and Sequencing. A part of the 16S rRNA mitochondrial gene was amplified using the primer pairs 16Sar-L (5′-CGC CTG TTT ATC AAA AAC AT-3′) and 16Sbr-H (5′-CCG GTC TGA ACT CAG ATC ACG-3′) as described by Palumbi.[12] The PCR was performed using a 25 μL reaction volume containing at least 10 ng of DNA extract, 0.2 μM of each primer, 0.2 mM of each dNTP, 1× GeneAmp PCR buffer (Applied Biosystem), 2.0 mM $MgCl_2$, and 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystem) in an ABI GeneAmp PCR system 9700 with the initial denaturation step at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 51 °C for 1 min, and then holding at 72 °C for 2 min. A final extension step of 10 min at 72 °C was included. For the COI gene, the primer pair COI-F (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3) and COI-R (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) and reaction conditions reported earlier[11,13] were used. The amplified products was visualized on a gel of 1.5% agarose in 1× TBE buffer with 5 μg/mL of ethidium bromide (GeneSee) and with an O'Gene Ruler 100 bp DNA ladder (Fermentas) as the size marker. The remaining PCR products were purified using DNA Clean and Concentrator-25 (Zymo Research) according to the supplier's instructions. Purified PCR products were directly sequenced using the ABI BigDye Terminator v3.1 cycle sequencing kit in an automatic ABI Prism 3130XL Genetic Analyzer. The resulting electropherograms were manually verified to check for any sequencing artifacts such as several ambiguous nucleotides at the beginning of the sequence read (near the primer binding site), dye blob, pull up, or background noise.

Data Analysis. All reference DNA sequences from the authentic samples, sequences retrieved from public databases NCBI GenBank or RefSeq, and the reference DNA sequence from a voucher specimen of *Concholepas concholepas* provided by NHM (Table 1) were subjected to FINS analysis using MEGA 4.1 beta software.[14,15] The genetic distance among sequences of samples was calculated using the nucleotide model of Tamura−Nei, and the phylogenetic relatedness among sequences was reconstructed with the neighbor-joining method. All positions containing alignment gaps and missing data were eliminated only in pairwise

Table 2. Tamura−Nei Distance Matrix of Different Neogastopods and Abalone Species[a]

	specimen (sample code or accession no.)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	*Hemifusus colosseus* (TD/BT/CRM/09001A)																				
2	*Hemifusus colosseus* (TD/BT/CRM/09001B)	0.000																			
3	*Hemifusus colosseus* (TD/BT/CRM/09001C)	0.000	0.000																		
4	*Melo melo* (TD/BT/CRM/09002A)	0.260	0.260	0.260																	
5	*Melo melo* (TD/BT/CRM/09002B)	0.260	0.260	0.260	0.000																
6	*Melo melo* (TD/BT/CRM/09002C)	0.262	0.262	0.262	0.002	0.002															
7	*Concholepas concholepas* (BMNH19990303)	0.199	0.199	0.199	0.256	0.256	0.259														
8	*Concholepas concholepas* (TD/HRM/09034)	0.200	0.200	0.200	0.254	0.254	0.257	0.000													
9	*Concholepas concholepas* (TD/HRM/09035)	0.199	0.199	0.199	0.256	0.256	0.259	0.000	0.000												
10	*Concholepas concholepas* (TD/HRM/09036)	0.199	0.199	0.199	0.256	0.256	0.259	0.000	0.000	0.000											
11	*Cymbium cymbium* (TD/BT/CRM/09003)	0.272	0.272	0.272	0.224	0.224	0.226	0.244	0.242	0.244	0.244										
12	*Cymbium cymbium* (TD/BT/CRM/09013)	0.275	0.275	0.275	0.224	0.224	0.226	0.244	0.242	0.244	0.244	0.002									
13	*Cymbium cymbium* (TD/BT/CRM/09017)	0.272	0.272	0.272	0.224	0.224	0.226	0.244	0.242	0.244	0.244	0.000	0.002								
14	*Cymbium tritonis* (TD/BT/CRM/09014)	0.261	0.261	0.261	0.212	0.212	0.215	0.252	0.250	0.252	0.252	0.074	0.072	0.074							
15	*Cymbium tritonis* (TD/BT/CRM/09004)	0.261	0.261	0.261	0.209	0.209	0.212	0.253	0.250	0.253	0.253	0.072	0.069	0.072	0.002						
16	*Cymbium tritonis* (TD/BT/CRM/09018)	0.261	0.261	0.261	0.209	0.209	0.212	0.253	0.250	0.253	0.253	0.072	0.069	0.072	0.002	0.000					
17	*Cymbium pepo* (TD/BT/CRM/09005)	0.264	0.264	0.264	0.207	0.207	0.209	0.255	0.253	0.255	0.255	0.076	0.079	0.076	0.033	0.031	0.031				
18	*Cymbium pepo* (TD/BT/CRM/09015)	0.264	0.264	0.264	0.207	0.207	0.209	0.255	0.253	0.255	0.255	0.076	0.079	0.076	0.033	0.031	0.031	0.000			
19	*Cymbium pepo* (TD/BT/CRM/09016)	0.267	0.267	0.267	0.207	0.207	0.209	0.255	0.253	0.255	0.255	0.076	0.079	0.076	0.033	0.031	0.031	0.000	0.000		
20	*Cymbium olla* (NC_013245)	0.274	0.274	0.274	0.212	0.212	0.214	0.265	0.263	0.265	0.265	0.080	0.078	0.080	0.061	0.058	0.058	0.065	0.065	0.065	
21	*Conus textile* (NC_008797)	0.229	0.229	0.229	0.257	0.257	0.260	0.246	0.246	0.246	0.246	0.256	0.256	0.256	0.248	0.251	0.251	0.254	0.254	0.254	0.267
22	*Lophiotoma cerithiformis* (NC_008098)	0.187	0.187	0.187	0.208	0.208	0.211	0.207	0.208	0.207	0.207	0.245	0.245	0.245	0.247	0.244	0.244	0.247	0.247	0.250	0.255
23	*Ilyanassa obsoleta* (NC_007781)	0.184	0.184	0.184	0.213	0.213	0.216	0.209	0.210	0.209	0.209	0.242	0.242	0.242	0.239	0.239	0.239	0.225	0.225	0.227	0.246
24	*Rapana venos* (NC_011193)	0.201	0.201	0.201	0.240	0.240	0.243	0.087	0.087	0.087	0.087	0.242	0.242	0.242	0.253	0.253	0.253	0.259	0.259	0.259	0.252
25	*Thais clavigera* (NC_010090)	0.215	0.215	0.215	0.244	0.244	0.241	0.091	0.091	0.091	0.091	0.268	0.268	0.268	0.265	0.265	0.265	0.268	0.268	0.268	0.263
26	*Cancellaria cancellat* (NC_013241)	0.221	0.221	0.221	0.203	0.203	0.206	0.196	0.196	0.196	0.196	0.226	0.226	0.226	0.225	0.228	0.228	0.214	0.214	0.214	0.234
27	*Haliotis rubra* (NC_005940)	0.405	0.405	0.405	0.382	0.382	0.385	0.426	0.426	0.426	0.426	0.393	0.393	0.393	0.374	0.378	0.378	0.381	0.381	0.381	0.402
28	*Haliotis asinina* (AY650173)	0.359	0.359	0.359	0.350	0.350	0.353	0.361	0.361	0.361	0.361	0.354	0.354	0.354	0.338	0.341	0.341	0.332	0.332	0.332	0.356
29	*Haliotis corrugata* (AY65017)	0.399	0.399	0.399	0.357	0.357	0.361	0.409	0.409	0.409	0.409	0.383	0.383	0.383	0.360	0.364	0.364	0.358	0.358	0.358	0.368
30	*Haliotis discu* (AY650174)	0.376	0.376	0.376	0.357	0.357	0.361	0.399	0.399	0.399	0.399	0.352	0.352	0.352	0.338	0.341	0.341	0.332	0.332	0.332	0.345
31	*Haliotis discus hannai* (EU636208)	0.380	0.380	0.380	0.364	0.364	0.368	0.400	0.400	0.400	0.400	0.356	0.356	0.356	0.337	0.341	0.341	0.332	0.332	0.332	0.349
32	*Haliotis fulgens* (AY650158)	0.381	0.381	0.381	0.342	0.342	0.345	0.402	0.402	0.402	0.402	0.377	0.377	0.377	0.356	0.360	0.360	0.346	0.346	0.346	0.358
33	*Haliotis gigantea* (AY650160)	0.372	0.372	0.372	0.361	0.361	0.365	0.395	0.395	0.395	0.395	0.363	0.364	0.363	0.341	0.345	0.345	0.335	0.335	0.335	0.357
34	*Haliotis iris* (AY650166)	0.400	0.400	0.400	0.349	0.349	0.353	0.418	0.418	0.418	0.418	0.381	0.381	0.381	0.363	0.367	0.367	0.358	0.358	0.358	0.371
35	*Haliotis midae* (AY650167)	0.395	0.395	0.395	0.384	0.384	0.387	0.422	0.422	0.422	0.422	0.390	0.390	0.390	0.360	0.364	0.364	0.370	0.370	0.370	0.384
36	*Haliotis ovina* (AY650154)	0.416	0.416	0.416	0.385	0.385	0.389	0.418	0.418	0.418	0.418	0.385	0.385	0.385	0.381	0.385	0.385	0.384	0.384	0.384	0.402
37	*Haliotis rufescens* (AY65016)	0.380	0.380	0.380	0.361	0.361	0.364	0.404	0.404	0.404	0.404	0.370	0.370	0.370	0.348	0.352	0.352	0.346	0.346	0.346	0.352
38	*Haliotis tuberculata* (AY650168)	0.353	0.353	0.353	0.339	0.339	0.343	0.389	0.389	0.389	0.389	0.376	0.376	0.376	0.341	0.345	0.345	0.347	0.347	0.347	0.368
39	*Albinaria coerulea - Outgroup* (NC_001761)	0.555	0.555	0.555	0.556	0.556	0.551	0.610	0.610	0.610	0.610	0.494	0.489	0.494	0.520	0.515	0.515	0.527	0.527	0.527	0.536
40	abalone China (TD/HRM/08013)	0.387	0.387	0.387	0.359	0.359	0.362	0.408	0.406	0.408	0.408	0.379	0.379	0.379	0.359	0.363	0.363	0.354	0.354	0.354	0.372
41	abalone Japan (TD/HRM/08004)	0.380	0.380	0.380	0.360	0.360	0.363	0.404	0.402	0.404	0.404	0.371	0.371	0.371	0.352	0.355	0.355	0.346	0.346	0.346	0.372
42	Chile (TD/HRM/08009)	0.199	0.199	0.199	0.256	0.256	0.259	0.000	0.000	0.000	0.000	0.244	0.244	0.244	0.252	0.253	0.253	0.255	0.255	0.255	0.265
43	USA (TD/HRM/08010)	0.227	0.227	0.227	0.289	0.289	0.292	0.272	0.273	0.272	0.272	0.303	0.303	0.303	0.305	0.305	0.305	0.305	0.305	0.308	0.310
44	SAT (TD/HRM/08011)	0.267	0.267	0.267	0.207	0.207	0.209	0.258	0.256	0.258	0.258	0.074	0.076	0.074	0.035	0.033	0.033	0.002	0.002	0.002	0.063
45	Senegal (TD/HRM/08014)	0.197	0.197	0.197	0.238	0.238	0.241	0.159	0.160	0.159	0.159	0.225	0.225	0.225	0.229	0.226	0.226	0.221	0.221	0.221	0.237

sequence comparisons (pairwise deletion option). The reliability of the tree was evaluated by means of a bootstrap test with 10000 replications.

Method Validation. In accordance with the SWGDAM guidelines, the following key areas of method validation were evaluated: (a) suitability of using partial 16S rRNA and COI DNA markers for differentiation between abalone and fraudulent species from other gastropod groups; (b) optimization of PCR performance; (c) sensitivity; (d) stability; and (e) reproducibility.

Market Survey. For the market survey, samples claimed to be "abalone" were seized by the Hong Kong Customs and Excise Department from some wholesale and retail outlets in the local market. A total of 28 samples claimed to be "dried abalone slices" and 1 canned abalone were examined in this study. DNA from all samples was extracted as described above.

RESULTS AND DISCUSSION

Development of the Genetic Identification Method by FINS. In this study, a method for identification of fake abalone species was established and validated following SWGDAM guidelines. Samples were identified either by a homology search against the established reference DNA sequences or by using the FINS approach to differentiate between the genuine and fake abalone species.

Table 2. Continued

21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44
0.205																							
0.215	0.127																						
0.242	0.206	0.189																					
0.269	0.212	0.195	0.076																				
0.219	0.206	0.199	0.192	0.217																			
0.430	0.450	0.385	0.428	0.430	0.370																		
0.400	0.407	0.361	0.390	0.395	0.338	0.130																	
0.408	0.427	0.369	0.411	0.409	0.357	0.086	0.102																
0.391	0.421	0.361	0.404	0.399	0.349	0.075	0.091	0.030															
0.396	0.422	0.362	0.405	0.399	0.353	0.077	0.091	0.030	0.004														
0.377	0.413	0.342	0.397	0.384	0.338	0.071	0.102	0.042	0.045	0.045													
0.381	0.426	0.357	0.405	0.404	0.349	0.079	0.097	0.039	0.008	0.012	0.052												
0.406	0.443	0.373	0.411	0.406	0.371	0.096	0.120	0.071	0.062	0.066	0.060	0.065											
0.417	0.449	0.394	0.431	0.426	0.382	0.072	0.101	0.089	0.071	0.073	0.083	0.074	0.082										
0.444	0.460	0.394	0.419	0.427	0.392	0.090	0.104	0.092	0.079	0.084	0.089	0.081	0.077	0.061									
0.391	0.424	0.368	0.405	0.403	0.353	0.087	0.091	0.017	0.016	0.016	0.040	0.025	0.070	0.085	0.086								
0.375	0.402	0.349	0.398	0.412	0.326	0.072	0.103	0.105	0.089	0.091	0.084	0.090	0.086	0.066	0.078	0.095							
0.517	0.543	0.562	0.572	0.596	0.576	0.590	0.546	0.565	0.547	0.552	0.591	0.548	0.567	0.578	0.560	0.558	0.560						
0.402	0.442	0.377	0.420	0.408	0.376	0.077	0.088	0.028	0.002	0.002	0.043	0.010	0.064	0.071	0.081	0.014	0.091	0.579					
0.394	0.434	0.374	0.416	0.405	0.377	0.080	0.091	0.030	0.004	0.004	0.045	0.012	0.066	0.073	0.084	0.016	0.093	0.579	0.004				
0.246	0.207	0.209	0.087	0.091	0.196	0.426	0.361	0.409	0.399	0.400	0.402	0.395	0.418	0.422	0.418	0.404	0.389	0.610	0.408	0.404			
0.267	0.192	0.210	0.251	0.260	0.289	0.483	0.423	0.450	0.431	0.432	0.442	0.433	0.461	0.477	0.471	0.457	0.440	0.603	0.447	0.439	0.272		
0.254	0.247	0.225	0.259	0.268	0.214	0.385	0.332	0.354	0.328	0.328	0.346	0.339	0.354	0.374	0.388	0.342	0.351	0.527	0.350	0.343	0.258	0.305v	
0.225	0.167	0.162	0.161	0.182	0.200	0.403	0.370	0.401	0.392	0.397	0.382	0.388	0.389	0.412	0.406	0.399	0.348	0.561	0.406	0.399	0.159	0.233	0.224

[a] Authentic dried seafood materials TD/HRM/08009−11 and -14 are conch slices from Chile, the United States, West Africa, and Senegal, respectively. TD/HRM/08004 and TD/HRM/08013 are dried abalones from Japan and China, respectively.

The suitability and discrimination power of the two marker genes, mitochondrial 16S rRNA and COI genes, for genetic identification of fake abalone were assessed on the basis of their ability to produce positive results in the PCR amplification and the evaluation of intraspecific and interspecific variations among species. The two marker genes were in the mitochondrial genome and can be amplified by corresponding universal primer pairs. Our results indicated that amplification of the 16S rRNA gene is more robust than that of COI. For the latter, it was difficult to obtain amplification results from *Cymbium cymbium* and in some processed abalone slices, perhaps due to low primer binding efficiency to the DNA of *C. cymbium* and DNA degradation in processed food, respectively, leading to its limited application in real cases. Therefore, only 16S rRNA was used for FINS analysis. COI served as an auxiliary DNA marker for genetic identification, because of the increasing use of the COI gene as the marker for barcoding data for species identification (e.g., BOLD database). According to previous publications,[16−19] the 16S rRNA gene is also useful for species-level differentiation. The distance analysis for 16S rRNA confirmed that intraspecific sequence variation is small when compared with interspecific variation. Table 2 shows that the sequence divergence within

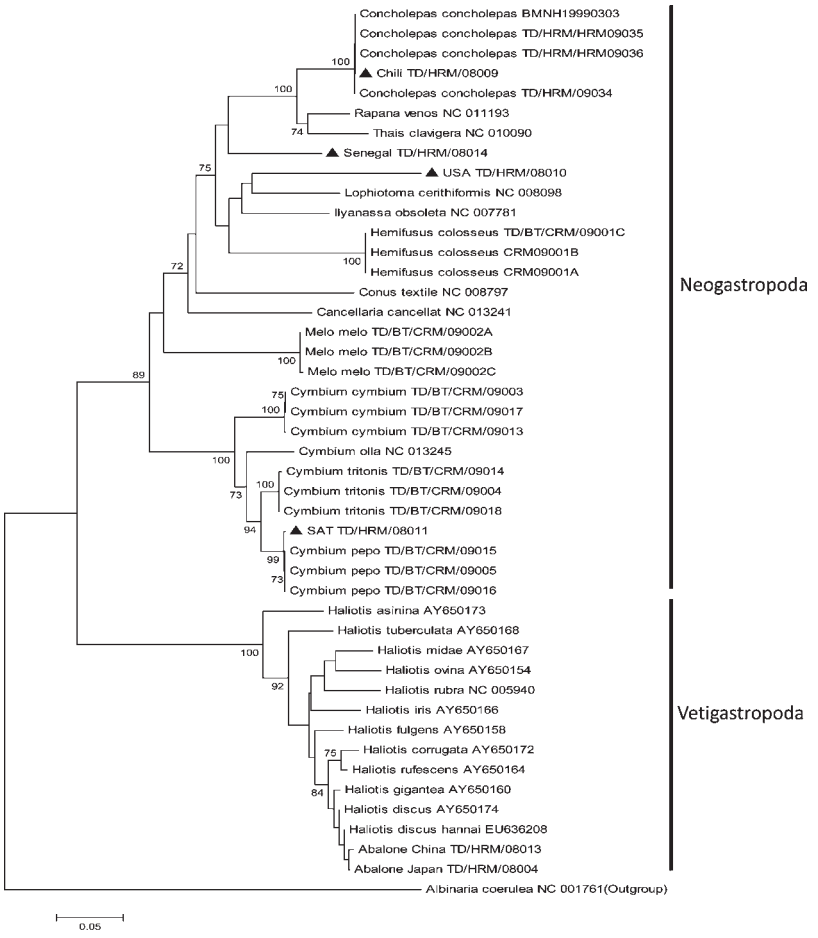

Figure 1. Phylogentic analysis of partial 16S rRNA partial sequences using Tamura−Nei distances and the neighbor-joining method. Bootstrap values >70 are shown in branches. Species of Neogastropoda are clearly distinguished from Vetigastropoda, that is, abalone (*Haliotis*) species. "▲" indicates authentic dried seafood material. TD/HRM/08009−11 and -14 are conch slices from Chile, the United States, West Africa, and Senegal, respectively. TD/HRM/08004 and TD/HRM/08013 are dried abalones from Japan and China, respectively.

Cymbium species is minor (0−0.2%) as compared to divergences at the interspecific level, such as among the four *Cymbium* species (mean of 3.1−8.0%), that is, *C. cymbium*, *C. pepo*, *C. tritonis*, and *C. olla*. From the result of FINS analysis using mollusc species listed in Table 1, the genetic distance between *Haliotis* and fake abalone species from the genera *Cymbium*, *Melo*, *Hemifusus*, and *Concholepas* is up to 35−40%, whereas the genetic distance among the *Haliotis* species is up to 17.5%. Suspected fake samples can be identified either by performing a pairwise alignment with reference DNA sequences for matching identity or by phylogentic analysis to look for closely related species. To assess the reliability and feasibility of the latter method, the reference set of 16S rRNA sequences and several authentic dried samples were used for a phylogenetic FINS analysis. As shown in Figure 1, the cultured abalone (*Haliotis*) species are clearly differentiated from all species of Neogastropoda with high bootstrap support (100%). Dried gastropod slices from Chile and West Africa were shown to be of *Concholepas concholepas* and *Cymbium pepo*, respectively. For the dried slices from the United States and Senegal, the identity could not be ascertained, but they are included in Neogastropoda with high bootstrap support. For our purpose, identification to species level is not essential. The main concern is to distinguish genuine abalone from fake abalone species, which are neogastropods, and in this our method succeeds. The certainty of the assignment of a sample to a specific clade in a phylogenetic tree can be assessed by the bootstrap confidence test.[20] This is one of the most widely used methods to assess the reliability of an inferred tree and the probability that a phylogenetic estimate represents the true phylogeny.[21] It has been reported that bootstrap proportions ≥70% usually correspond to a probability ≥95% that the corresponding cluster is real.[22] In Figure 1, the bootstrap values obtained for the clusters of sequences from the species of interest were always ≥70%, which indicated that the clustering is reliable and the assignment correct.

Method Validation. Amplification from molluscan tissues can be difficult, as these tissues have a high mucopolysaccharide content leading to low DNA quality. To overcome this, PEG with molecular weight >6000 was added to the extraction buffer to a final concentration of 1% for removal of mucopolysaccharides. DNA quality was found to be significantly improved with OD 260/230 increased from 0.7−1 (before the addition of PEG) to 2.2−2.4 (after the addition of PEG). This means that the copurified contaminants were removed by the addition of PEG in the extraction step. For the optimization of PCR performance and assessment of the PCR specificity, different magnesium concentrations (1.5 and 2.0 mM) were evaluated using two reference neogastropods, *Cymbium cymbium* and *C. pepo*.

Table 3. Market Survey Results

code	product claim	detected species	similarity range %
A1	dried abalone slice (South Africa)	*Cymbium pepo*	100
A2	dried abalone slice (North Africa)	*Cymbium pepo*	100
A3	dried abalone slice (Japan)	*Cymbium pepo*	100
A4	dried abalone slice (South Africa)	*Cymbium pepo*	100
A5	dried abalone slice (Chile)	*Cymbium pepo*	100
A6	dried abalone slice (South Africa)	*Cymbium* sp.	97.4−97.7
A7	dried abalone slice (Australia)	*Cymbium cymbium*	99.7−100
A8	dried abalone slice (Australia)	*Cymbium* sp.	97.4−97.7
A9	dried abalone slice (Japan)	*Cymbium cymbium*	99.7−100
A10	dried abalone slice (Australia)	*Concholepas concholepas*	100
A11	dried abalone slice (Australia)	*Cymbium cymbium*	99.7−100
A12	dried abalone slice (Australia)	*Cymbium cymbium*	99.7−100
A13	dried abalone slice (Australia)	*Cymbium cymbium*	99.5−99.7
A14	dried abalone slice (Chile)	*Concholepas concholepas*	100
A15	dried abalone slice (South Africa)	*Cymbium pepo*	99.7
A16	dried abalone slice (South Africa)	*Cymbium pepo*	100
A17	dried abalone slice (South Africa)	*Cymbium pepo*	100
A18	dried abalone slice (South America)	*Cymbium pepo*	99.7
A19	dried abalone slice (Japan)	*Cymbium* sp.	97.7−97.9
A20	dried abalone slice (Japan)	*Cymbium cymbium*	99.7−100
A21	dried abalone slice (Japan)	*Cymbium pepo*	100
A22	dried abalone slice (Australia)	*Cymbium pepo*	100
A23	dried abalone slice (Chile)	*Cymbium* sp.	97.4−97.7
A24	dried abalone slice (Australia)	*Cymbium* sp.	97.4−97.7
A25	dried abalone slice (Australia)	*Cymbium* sp.	97.4−97.7
A26	dried abalone slice (Japan)	*Cymbium* sp.	97.4−97.7
A27	dried abalone slice (Australia)	*Cymbium pepo*	100
A28	dried abalone slice (Africa)	*Cymbium* sp.	97.9
A29	abalone (New Zealand)	*Haliotis* sp.	99

Positive results were defined as PCR signal occurring at the correct fragment size of about 500−550 base pairs (bp) for the 16S rRNA gene and about 650 bp for the *COI* gene in agarose gel analysis and the amount of the PCR product being sufficient for the subsequent sequence analysis. The results indicated that PCR reactions with magnesium concentration of 1.5 mM produced weak signals in some samples, whereas 2.0 mM magnesium gave strong PCR signals for all samples. No evidence of nonspecificity was found. The sensitivity, in terms of the limit of detection (LOD), is defined as the lowest mass of DNA in a sample at which an authentic sample yields a PCR positive result with at least 95% confidence. The PCR LOD was assessed by analysis of consecutive dilutions (0.1, 1, and 10 ng) of the total genomic DNA obtained from four independent DNA extractions from the reference materials. The method gave high sensitivity with LOD of 10 ng of DNA template for both target genes 16S rRNA and COI, and no DNA sequence variation was observed among the independent replicates of these reference materials. The applicability of the method was defined as the ability to obtain positive results from DNA recovered from biological samples in the form of either the whole fresh molluscs or molluscs processed by canning or drying of sliced pieces. The primer pair for 16S rRNA gave good signals for all of the reference samples examined irrespective of the form of the sample, that is, no matter if it was fresh, dried, whole, or sliced. Reproducibility was assessed by conducting separate PCR reactions using the aforesaid reference materials four times and having the consistency determined by multiple sequence alignment with ClustalW or MEGA software. The results indicated that all sequencing data from the replicates are consistent.

Identification of Commercial "Abalone" Samples by FINS. The proposed authentication method was further evaluated with the samples taken from different retail outlets in the local market. These samples were successfully identified as genuine or fake using the FINS method. The results are shown in Table 3 and Figure 2. The abalone species and all neogastropod species are clearly differentiated with high bootstrap values of 100 and 93%, respectively. Only one product, canned abalone (A29), was found to be a *Haliotis* species as claimed. The rest of the samples tested were identified as neogastropods. Twelve "abalone slice" samples (A1−5, A15−18, A21−22, and A27) were found to be of *Cymbium pepo*, while six "abalone slice" samples (A7, A9, A11−13, and A20) were found to be *Cymbium cymbium*. Among these samples available in the market, the identity of eight products (A6, A8, A19, A23−26, and A28) can only be determined to genus level, as *Cymbium* species. Sequence divergence within species is usually <2%; in other words, samples with genetic distance >2% may belong to other species, although of course this value varies with the choice of gene markers and evolutionary history of the organisms. For examples, the nucleotide divergence of geographic samples within abalone species in Thailand is 0−0.47% within *Haliotis asinina*, 0−1.07% within

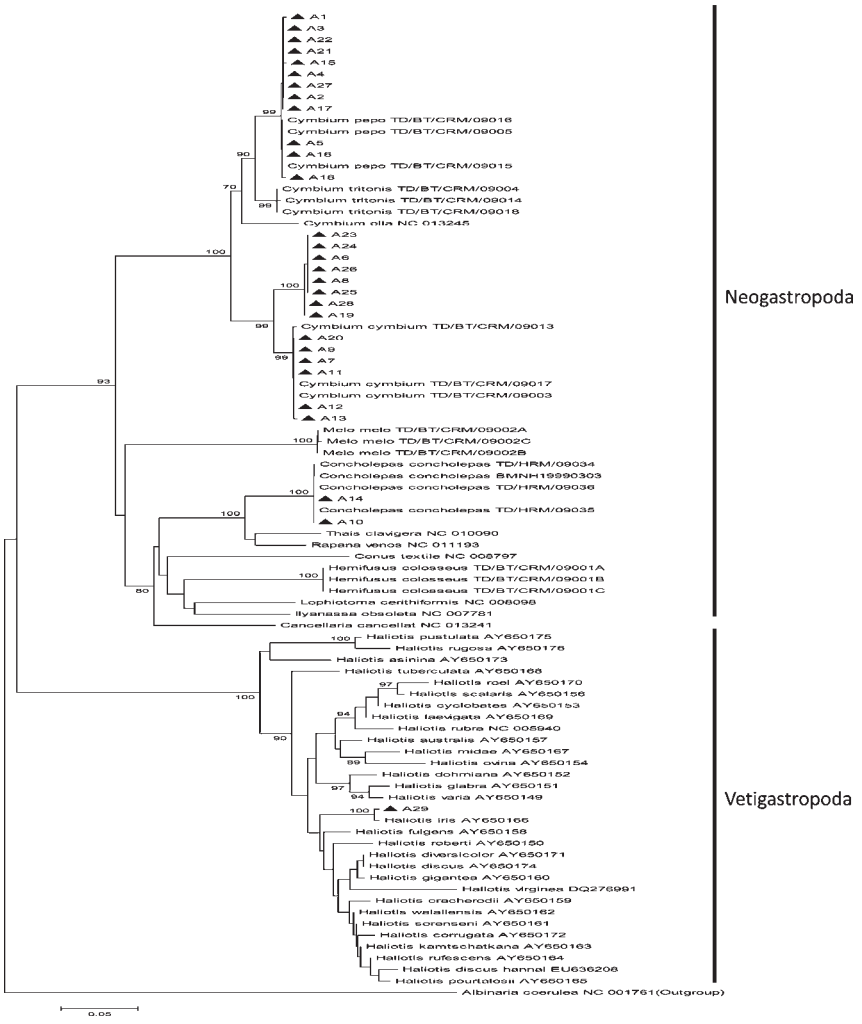

Figure 2. Identification of commercial "abalone" samples by FINS. Bootstrap values >70 are shown in branches.

H. ovina, and 0−0.02% within *H. varia*, based on 18S and 16S rRNA.[23] It has been reported that there is 0.12−1.3% sequence divergence between COI haplotypes of the pearl oyster *Pinctada mazatlanica*.[24] The eight unknown market samples (A6, A8, A19, A23−26, and A28) have genetic distances from *Cymbium cymbium* of 2.3−2.6% and grouped together with other *Cymbium* species. On the basis of the aforementioned sequence divergence within the same species (usually not more than 2%) and our previous results showing the genetic distance among reference *Cymbium* species (i.e., *Cymbium cymbium*, *C. pepo*, *C. tritonis*, and *C. olla* in this study ranging from 3.1 to 8.0%), these market samples were considered as belonging to the genus *Cymbium* and being closely related to *Cymbium cymbium*. This reveals that a sample could be identified to the level of the genus *Cymbium* whenever the similarity is >2% but ≤8%. In summary, the results of our market survey showed that all suspected fake samples labeled as "abalone slice" for sale are fraudulent. They were found to be of either *Cymbium* species (93%) or *Concholepas concholepas* (7%). The genus *Cymbium* is restricted to West Africa and southwestern Europe,[25] whereas *Concholepas* is endemic to Chile and Peru.[26] To the best of our knowledge, this is the first report of *Cymbium* species being used for making fake abalone slices to be sold in Hong Kong.

Conclusion. The FINS approach was proposed by Barlett and Davidson to estimate the genetic distances between groups of reference sequences and an unknown sample as a means of species identification.[9] Subsequently, Brodmann's research group reported the combination of DNA sequencing and the basic local alignment search tool for identifying unknown game species.[27] Since then, these methods have been widely applied for species identification of different meat and seafood products.[6−9,27,29−32] An analytical test result, based on the comparison with a reliable reference DNA sequence database established from a collection of reference specimens, is highly recommended whenever court proceedings are required. However, it is always difficult to collect sufficient reference material for the case as the full range of animals that are used as counterfeits by dishonest traders cannot be predicted. Reference sequences retrieved from public databases such as GenBank and BOLD are a good alternative as they contain millions of sequences from various animal and plant species. Whenever possible, we recommend using NCBI Reference Sequence (RefSeq) and then sequences from voucher specimens that have been certified by recognized authorities or experts. It is recommended that the method should be validated using control materials before application to case work. In this study, we successfully demonstrated the integration of voucher

samples certified by recognized authorities and reference DNA sequences retrieved from public databases for species determination so as to provide strong evidence to support our findings.

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DISCLOSURE

The contents of this paper do not necessarily reflect the views of the Government of the HKSAR, nor does mention of trade names or commercial product constitute endorsement or recommendations of use.

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