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Molecular identification of croaker dried swim bladders (maw) on sale in Hong Kong using 16S rRNA nucleotide sequences and implications for conservation

Cynthia Darta Tuuli, Yvonne Sadovy de Mitcheson, Wai-Chuen NG *

The Swire Institute of Marine Science, The University of Hong Kong, Cape d'Aguilar Road, Shek O, Hong Kong

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ABSTRACT

Hong Kong is a major import and transhipment centre for seafood inclusive of a wide range of dried products. Dried fish swim bladder (commonly referred to as 'maw') has long been a significant and valuable component of dried seafood sold in the city. Although the species and provenance of swim bladder are poorly documented the major target taxon involved has historically been the croakers (Sciaenidae). This study is the first to examine the possible provenance and species supplying dried fish swim bladder on sale in Hong Kong using DNA barcoding based on the partial sequence of the 16S rRNA gene. A reference sequence database was constructed from available Genbank fragments and sequences obtained from freshly identified fish specimens. Seventy-nine individual dried fish swim bladders, consisting of four commonly sold morphotypes, were examined with sequences successfully obtained from ~46% of the samples. Five taxa were identified, four of which matched known species in the sequence database, including three sciaenids and one latid. Confidence in species identifications was high due to low within-group divergence (0.0–1.3%) and high between-group divergence (≥2.9%). None of the identified species is local to Hong Kong waters and none are cultured. Several croakers exploited for swim bladder are depleted or threatened, two of them globally endangered. Interest in fish swim bladder in Chinese markets remains high and it has recently become a replacement for sharks' fin in banquets with decline in fin use. These findings highlight the value of barcoding for species verification of processed fish swim bladders and its potential application in the monitoring of their international trade. The findings also highlight data gaps for species coverage in the available DNA sequences and the need for better trade monitoring of dried swim bladder.

1. Introduction

Hong Kong has one of the highest seafood consumption rates in the world, second amongst developed countries and eleventh globally (FAO, 2012). The city is also the world's largest dried seafood importer and re-exporter (Clarke, 2002). This massive import and export trade, particularly with Mainland China, involves a large number of fish and invertebrate species and is officially documented by the Census and Statistics Department (CSD) of the government of the Hong Kong Special Administrative Region (HKSAR, Fig. 1). In 2012, the CSD Harmonized Codes included many categories of dried commodities. Most codes, however, cover multiple species or general categories such as frozen or pickled or dried products. Hence trends over time, or volumes and provenance of individual species in trade, are poorly understood.

One highly valued dried seafood item in Hong Kong and Mainland China is the dried fish swim bladder (commonly referred to as 'maw'.). Locally known as "花膠" (pronounced "Fa Gaau"), fish swim bladder is favoured for its supposed medicinal benefits and for use as a tonic and as a food base. It is highly regarded for its rich collagen content, as a specialized adhesive, and for clarification of wine and beer (Hickman, 1999). The processing of dried swim bladder involves its removal from the fish and a longitudinal cut. It is washed with water to remove blood vessels, then placed on a mat and regularly turned to dry in the sun. After partial drying, the bladders are smoked in a sealed container with sulphur for several hours, then shaped flat for sale. Some details of dried swim bladder preparation are trade secrets.

Little is known of the fisheries associated with the collection of fish swim bladder but its use has a long history in China. Lin

* Corresponding author. Fax: +852 2809 2197.
E-mail addresses: dfatchu@yahoo.com (C.D. Tuuli), yjsadovy@hku.hk (Y.S. de Mitcheson), chueneugene@yahoo.com (N. Wai-Chuen).

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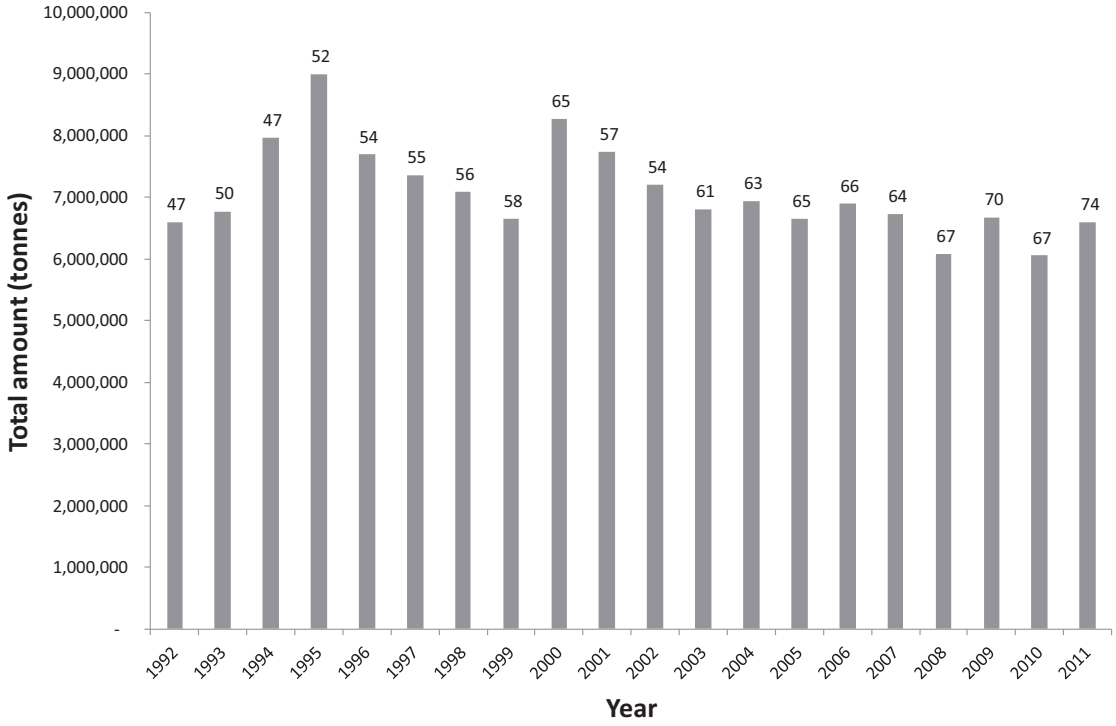


Fig. 1. Volume of trade data for "dried fish" recorded by Census and Statistics Department of the government of the Hong Kong Special Administrative Region from 1992 to 2011. Number above each bar represents the number of countries recorded in the trade data.

(1939) wrote a detailed paper on the trade, chemical composition, preparation, food and other use (isinglass; glue), and morphology of dried swim bladder. His account listed several croaker species (blackspotted croaker, *Protonibea diacanthus*, large yellow croaker, *Larimichthyes crocea*, Chinese bahaba, *Bahaba taipingensis*, white-flower croaker, *Nibea albiflora*) as the main sources of swim bladder sold in Hong Kong. Most swim bladders at the time were imported from India, Sri Lanka, Myanmar, Malaysia, Cuba, Indo-China, Thailand, Philippines and South America although the species were evidently not documented. Larger and thicker swim bladders are more highly favoured in trade as are those of certain species. For example, at the time of Lin's account, dried swim bladder of the Chinese bahaba and other croakers, as well as of the conger-pike (distinctively elongated) pufferfish and catfish (both triangular and distinct in morphology from those of croakers) were also important.

Dried seafood is a broad seafood category under the CSD that is regularly imported into Hong Kong. The category comprising fish skin, bones and fillets, whole, skinless, headed and gutted dried fish, and fish swim bladder (Clarke, 2004), is listed under Harmonized Code System No. 03055990—and refers to 'Fish, dried, whether or not salted, but not smoked, 'nesoi", This code aggregates the imports of a wide range of species, from dried anchovies and dried conger pike eels to other dried fish and fish swim bladder. From 2002 to 2011, mean imports of all dried seafood under this category were steady with a mean of 7089 tonnes (standard error = 163 tonnes). In 2004 dried seafood imports came from 56 countries, with Bangladesh, Vietnam and Mainland China accounting for over 50% of the volume (Kan, 2005). Conover and Dong (1998) determined from a market survey that the majority of the dried fish category was comprised of swim bladder, as indicated also by Clarke (2002). Dried swim bladder was only separated from other dried fish under the Harmonized Code System as of January 2015. Data for the first eight months of imports of swim bladder totalled 3438 tonnes, a significant portion of the mean annual imports of dried fish.

A major taxon sourced for swim bladder is the family Sciaenidae (the croakers). Croaker bladders are particularly favoured and fetch the highest prices; however those of eel (Anguillidae and Congridae) and catfish (Ictaluridae) families are also sold (Lin, 1939; Clarke 2002; Tuuli, 2010). Market pressures on croakers for food and for their swim bladder, a life history that makes many species of this family vulnerable to overfishing (e.g., longevity and aggregation-spawning), combined with a lack of management for most croaker fisheries, have led to the overexploitation of many species; several are now threatened with extinction (Sadovy and Cheung, 2003; Clarke, 2004; Liu and Sadovy de Mitcheson, 2008). Extremely high prices are paid for several large species that are now rare. In 2012, for example, one large swim bladder from the Chinese bahaba (also known in English as the giant yellow croaker or yellow-lipped croaker), endemic to east Asia and which is critically endangered (Ng and Cheung, 2006) and is protected in China, fetched almost half a million US$ in Mainland China in 2012 (Moore, 2012) This species has been fished close to extinction because of heavy exploitation for its swim bladder (Sadovy and Cheung, 2003). Another croaker, *Totoaba macdonaldi*, the totoaba, which has a swim bladder similar to that of the bahaba, is heavily sought for its swim bladder for Chinese markets where it also fetches high prices and for which illegal sale was recently reported in Hong Kong (Greenpeace, 2015). This protected species is on CITES (Convention on International Trade in Endangered Species) Appendix I, so all international trade is illegal. It is also listed as critically endangered on the IUCN Red List (Findley, 2010). Nonetheless, it is so valuable that poaching continues (Spagat, 2013).

The paucity of information on the species and regarding the countries of origin of dried swim bladder represent major gaps in our understanding of important aspects of a large and valuable seafood trade, and one that is threatening several species with extinction unless controlled for sustainability. At the retail and wholesale levels in Hong Kong traders use texture, size, form and thickness of swim bladder, i.e. primarily morphological characteristics, to determine price and assign market names (Lin, 1939; Clarke,

2002). The physical similarities of many bladder types mean that different species may be sold under the same trade names with a fair degree of interchangeability. There is no standardized naming system for swim bladders and there are many categories. With the exceptions of the highly valued swim bladders discussed above, names can be assigned according to the fish involved, shape, process method or the country of origin. Traders tend to report "origin" as the place from which they imported the product, while customs data distinguish 'country of origin' from 'country of consignment'. Although it may sometimes be difficult, using CSD trade data, to distinguish major source countries from trade 'entrepots' this is not considered to be a major problem for dried swim bladder since the introduction of a code, in January 2015, for this commodity has allowed for a much clearer indication of recent origin. Data from January to August 2015 show 7 source/entrepot countries (in order from highest volume: Brazil; Uganda; India; Tanzania; China; Guyana and Indonesia) that exported more than 100 tonnes each during the 8 months period, with total monthly imports to Hong Kong, from all 67 countries ranging from 140 to 340 tonnes. Singapore, often a major entrepot for seafood entering Hong Kong, accounted for only a negligible (<1%) percentage of trade in the first eight months of 2015. In this study, our interest is in species and provenance of dried fish swim bladder entering Hong Kong's retail sector.

An increasingly employed molecular approach to identify marine species is the examination of diagnostic nucleotide positions in DNA sequences, e.g. the mitochondrial 16S rRNA (16S) gene and cytochrome c oxidase subunit I (COI) gene (Ward et al., 2009; Lakra et al., 2009). Among these genes, the 16S sequence is commonly applied in the phylogenetic analysis of certain families of Perciformes (Lakra et al., 2009). For sciaenids, the family that is a major source of dried swim bladder, the 16S gene shows relatively high nucleotide divergence and was regarded as appropriate to describe family interrelationships (Vinson et al., 2004; Lakra et al., 2009). The sequence has also been employed in molecular identification of fish larvae (Díaz-Viloria et al., 2013) and fish eggs (Kawakami et al., 2010) due to its ability to discriminate among fish species. In this study, with the collection of sequences from 85 sciaenid species, we evaluated the genetic divergence and performance of the 16S gene in species identification for dried swim bladders.

It is of considerable interest to better understand the species that supply dried fish bladder to the Hong Kong market for both fishery and conservation concerns as well as to inform traders and consumers. This study is a first step towards documenting the most commonly used fish species currently supplying swim bladder sold in Hong Kong's dried seafood retail markets. The initial focus was on croakers because this taxon is particularly favoured and reported to make up a high proportion of dried seafood (e.g., Clarke, 2002). There are also concerns over the conservation status of several croakers known to supply dried swim bladder. The study involved initial categorization of swim bladder based on external morphological characteristics and according to trade categories as indicated by traders. Species were then characterized using mitochondrial 16S rRNA gene sequences which were compared against a sequence database of identified fish species, either lodged in GenBank or were available from freshly identified fish specimens.

2. Materials and methods

2.1. Sample collection

From September 2007 to January 2008 a study was conducted of dried seafood shops at Sai Ying Pun, Hong Kong, the major area for dried seafood in the city, to identify common types of fish swim bladder on retail sale. Fifteen shops out of about 90 selling dried swim bladder during the study period were visited and shopkeepers were asked to sell samples of the most common dried 'croaker' swim bladder that they had in stock and to identify its country of origin, and type, i.e. putative species. A total of 79 swim bladder samples identified as derived from croakers, commonly sold by traders, was purchased for laboratory analyses. The purchased specimens were examined in the laboratory and classified into morphotypes based on morphological characters, including size, texture and thickness (Table 1). Four morphotypes were identified. Source locations indicated by traders are presented wherever provided (Table 1). Whole specimens of thirty individual bladders were retained as voucher specimens and photographs taken of morphologically representative examples.

2.2. DNA extraction, PCR amplification and sequencing

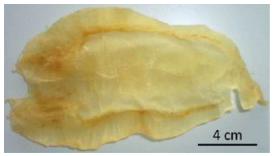
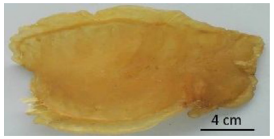
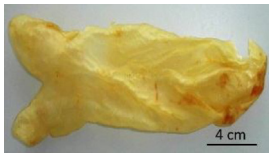
For each sample, genomic DNA was extracted from 25 mg swim bladder tissue using the Qiagen QIAamp® DNA Mini Kit (Catalogue no. 51306). Extracted DNA was visualized by electrophoresis in a 1% agarose gel stained with 0.4 ng/ml of ethidium bromide in 1× Tris-acetate-EDTA (TAE) at 100 V for 30 min. A GeneRuler™ 100 bp DNA Ladder Plus (Fermentas) was then used as standard size. All DNA samples were stored at −20 °C pending amplification and sequencing.

Amplification was performed for the 16S rDNA region (16S) of mitochondrial DNA (mtDNA). Primers used for 16S gene amplification followed were 16SAR (5′-CGCCTGTTTATCAAAAACAT-3′) and 16SBR (5′-CCGGTCTGAACTCAGATCACGT-3′) (Palumbi et al., 1991). Samples were amplified in 25 μL reactions containing ~50 ng of genomic DNA, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 0.2 mM each dNTP, 2 mM $MgCl_2$, 20 mM each primer, and 1 unit of *Taq* DNA polymerase (Promega). Polymerase chain reaction (PCR) was performed using DNAEngine© Peltier Thermal Cycler (BIO-RAD) with temperature profile of an initial denaturation at 94 °C for 3 min, followed by 25 cycles of 94 °C (1 min), 50 °C (1 min) and 72 °C (2 min), and a final extension step of 72 °C (10 mins). The amplicons were visualized using 1.5% agarose gel. The PCR product (~500 bp) was subsequently sequenced with the same pair of primers as for the amplification, using ABI 3730 sequencer (Applied Biosystems) at Tech Dragon Ltd Co., Hong Kong.

2.3. Database sequences and species verification

Species identities of the dried swim bladder specimens were evaluated based on reference database sequences collected from three sources: (1) sequences of each individual bladder were subjected to BLAST (Basic Local Alignment Search Tool) search in the GenBank. BLAST is an algorithm for comparing sequence information such as DNA sequences thereby enabling identification of matching sequences from a sequence library. The top three matched taxa from GenBank with the associated percentage similarities were recorded, and the 50 closest matches for each queried sequence were retrieved as database sequences; (2) 16S sequences for all available sciaenid species were retrieved from GenBank; (3) additional (to GenBank) confirmed-species sequence reference materials were available from South America from the Federal University of Para (Universidade Federal do Para), Brazil, and a further 4 species were collected in Hong Kong (this study). Before assessing the dried swim bladder, divergence of the database sequences was evaluated with alignment of the sequences using Clustal W (Thompson et al., 1994), followed by computation of pairwise distances using Kimura 2-parameter model (Kimura, 1980) in MEGA 6 (Tamura et al., 2013). The level of genetic variation within species (intraspecific), between species (interspecific) and between gen-

Table 1
Morphological characters, source location as claimed by trader, number of specimens collected and analyzed for the maw types in this study.

Maw type	Local name	Morphological characters/claimed source location	Photo	Price (US$/kg)	Number specimens	Number with high quality DNA	Number successful PCR	Number successfully sequenced
A	“白花” (“Ba Fa”)	≤0.2 cm thick, small and very light in colour; among the most common maw morphotypes available on sale. Maw is coarse in texture and tapers at one end. Morphotype is very light in colour. Claimed source location: local waters		40–90	32	20	15	13
B	“花膠” (“Fa Gaau”)	≤ 0.5 cm thick, size variable, texture smooth and shape ovoid and very pointed at one end. Morphotype is light in colour with 2 protrusions anteriorly. Claimed source location: Peru		500–800	29	18	15	15
C	“鮸魚” (“Man Yue”)	0.5-1.5 cm thick and dark in colour. Slightly rounded in shape with constrictions at one end and bulging at other end, smooth in texture. Claimed source location: India		1800–3000	12	8	4	4
D	“花膠” (“Fa Gaau”)	≤0.5 cm thick, light in colour compared with ‘man yue’ (above); has a very thick midsection and can be round or cut open and flat. Claimed source location: unknown		120–180	6	4	4	4
				Total	79	50	38	36

era (intergeneric) was assessed to evaluate the efficiency of species discrimination.

Species verification of swim bladder samples was carried out with alignment of sample sequences with the database sequences using ClustalW (Thompson et al., 1994) and clustered in MEGA 6 (Tamura et al., 2013) using a neighbour-joining (NJ) tree with Kimura 2-parameter distance (Kimura, 1980). The pairwise deletion option was selected to account for missing sequence information between each compared specimen. The tree was bootstrapped (1000 iterations) to assign confidence levels to each branch of the tree.

3. Results

3.1. Sample collection and morphotypes

Four fish swim bladder morphotypes (classified by their external morphology as A–D), sold as 'croaker maw' were commonly sold in Hong Kong's dried seafood sector during the study period (Table 1). In some cases the trader ascribed a particular croaker species name; in others, the generic name "Fa Gaau" was used for different morphotypes. Among the 79 specimens, successful sequences were obtained for 36 specimens from the four morphotypes after repeated trials with different extraction and PCR conditions (Table 1).

3.2. Sequence reference database

Distinct match results between swim bladder types A–D were obtained by BLAST search against the NCBI database. Sequences from morphotypes A–C showed the closest matches to sciaenid species: a total of 157 sequences from 54 species were acquired from the database and all the species were from Sciaenidae. The maximum identity of the match ranged from 87 to 100%. The sequence database was further enriched with the inclusion of 16S sequences from all available sciaenid species (85) from the NCBI database. Moreover, 15 sequences from 15 sciaenid species were obtained from the Universidade Federal do Para, Brazil and 6 sequences from 4 sciaenid species amplified from Hong Kong samples. The new sequences from Brazil and Hong Kong did not add new sciaenid species to the reference database but did provide more sequences. In summary, a total of 211 sequences from 85 species (41 genera) of Sciaenidae was compiled.

For morphotype D, the top 50 hits from BLAST search resulted in 33 species from 4 families (Latidae, Carangidae, Xiphiidae and Scophthalmidae). None of these matched sequences belonged to Sciaenidae (croaker). The maximum identities ranged from 84 to 100%, with highest match to species of latid.

3.3. Divergence of 16S sequences

Among the database sequences for Sciaenidae, 209 sites of the 437 base-pair (bp) fragments were variable within and among species, with 197 being phylogenetically informative. The K2P divergences of 16S sequences were evaluated and the genetic variability among the database species is shown (Fig. 2) to evaluate for the efficiency of species differentiation. An increase in genetic variation was observed with increase in taxonomic level (i.e., K2P distance increases from species to genus level, and so on). Distribution of intraspecific (0.0–1.6%) and interspecific (1.2–10.0%) divergence revealed low overlap between the two levels (Fig. 2). Intergeneric divergence ranged from 1.2 to 26.8%, reflecting a relatively large overlap with interspecific variations. For Latidae, there was no overlap between the intra- (0%) and inter-specific (3.4–4.8%) divergence from the three available latid species (i.e., *Lates calcarifer*, *L. niloticus* and *L. japonicus*). Forty-five of the 308 bp fragment were variable, with all of them being phylogenetically informative.

3.4. Species verification

Species matched by BLAST and tree-based methods for each swim bladder specimen showed good agreement for three of the four morphotypes (Table 2). No confident matches were obtained for all 13 specimens of morphotype A. Low percentage similarity (91–92%) with *Pogonias cromis* was obtained from BLAST which was too low for a reliable match. NJ tree grouped all 13 specimens in the same cluster (Fig. 3a) with low within-group divergence (0–0.5%, Table 3), suggesting they all belong to same species; however no species-match was possible. Morphotype A was labelled by traders as "Ba Fa" ("白花"), a Chinese common name for the sciaenid *Nibea albiflora*. However, analysis revealed a negative match of morphotype A to the *Nibea albiflora* sequence in the database.

Two matched species were identified among the 15 specimens of morphotype B, with specimen B01 matched with *Cynoscion microlepidotus* with the other 14 specimens matching with *Cynoscion acoupa*. All four specimens of morphotype C matched with *Otolithoides biaurithus*. For morphotype D, the four specimens matched with species in the family Latidae (Fig. 3b). Specimen D4 clustered with the Nile perch *Latus niloticus*, while the other three specimens formed a cluster close to the same species with low genetic divergence (1.3%), which likely represented a within-species divergence. All species matches were supported by both high percentage similarities in BLAST (99–100%, Table 2) and clean cluster (Fig. 3a and b) with low within-group divergence (0–1.3%, Table 3). Overall, consensus species identification was obtained for 20 of the 36 specimens (Table 2).

4. Discussion

This study is the first attempt to determine the major fish species supplying swim bladder to the dried seafood market in Hong Kong using molecular techniques, and to identify their possible countries of origin, with a particular focus on croakers. The overall volume of the trade, morphological similarities of several morphotypes of swim bladders commonly sold as croakers, and the management or conservation concerns for several croaker species historically valued for their dried swim bladders mean that a better understanding of species supplying the trade is both timely and important. There is a dearth of detailed information on provenance and volumes by species traded for their swim bladders, or on the overall economic significance of the dried swim bladder trade. The value is known to be very high for certain species, especially larger croakers, and in recent years the lucrative market has fuelled interest to target the more highly valued species and sizes (e.g., loc. cit.; Mudur 2012).

This study collected four major morphotypes of dried swim bladder commonly found on retail sale, labelled as croakers, from a random selection taken from Hong Kong dried seafood shops. We attempted to resolve taxonomic identities using the partial sequence of the 16S rRNA gene. Five taxonomic clusters were identified, four of which matched known species in the sequence database; three in the family Sciaenidae and one in the family Latidae.

4.1. DNA barcoding on dried fish swim bladder

The discriminatory power of DNA barcoding depends on two major factors, the level of sequence divergence, and the coverage of the database and accuracy with which species are identified. (Hajibabaei et al., 2007; Ward et al., 2009). The extent of, and separation between, the intra- and interspecific divergence govern

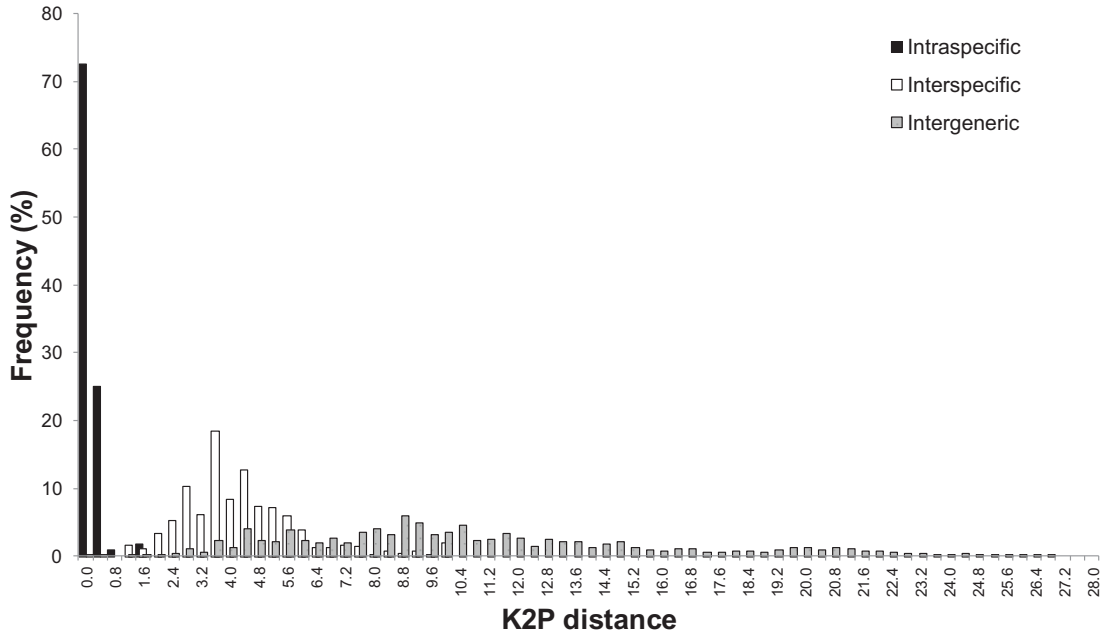


Fig. 2. Frequency distribution of interspecific and intraspecific genetic divergence in 16S sequences of sciaenid species. Total number of comparisons: 120 intraspecific and 25,758 interspecific pairs across 85 Sciaenidae species (228 individuals). Divergences were calculated using Kimura 2-parameter (K2P) model.

Table 2
Fish maw species verification. The best three matched species from BLAST (with percentage similarity), the tree-based identification (with percentage bootstrap) and the consensus species identification for the fish maw samples.

Maw sample	BLAST (% similarity)						Tree-based method (% bootstrap)		Consensus species ID
	1st match	%	2nd match	%	3rd match	%	Matched species	%	
A02	*Pogonias cromis*	92	*Cheilotrema saturnum*	92	*Miichthys miiuy*	92	N.A.	100	N.A.
A04	*Pogonias cromis*	91	*Cheilotrema saturnum*	92	*Michthys muiiy*	91	N.A.	100	N.A.
A05	*Pogonias cromis*	92	*Cheilotrema saturnum*	92	*Michthys muiiy*	92	N.A.	100	N.A.
A06	*Pogonias cromis*	92	*Cheilotrema saturnum*	92	*Michthys muiiy*	92	N.A.	100	N.A.
A09	*Pogonias cromis*	92	*Cheilotrema saturnum*	92	*Michthys muiiy*	92	N.A.	100	N.A.
A10	*Pogonias cromis*	92	*Cheilotrema saturnum*	92	*Michthys muiiy*	92	N.A.	100	N.A.
A12	*Pogonias cromis*	92	*Cheilotrema saturnum*	92	*Michthys muiiy*	92	N.A.	100	N.A.
A17	*Pogonias cromis*	92	*Cheilotrema saturnum*	92	*Michthys muiiy*	92	N.A.	100	N.A.
A19	*Pogonias cromis*	92	*Cheilotrema saturnum*	92	*Michthys muiiy*	92	N.A.	100	N.A.
A23	*Pogonias cromis*	92	*Cheilotrema saturnum*	92	*Michthys muiiy*	91	N.A.	42	N.A.
A26	*Pogonias cromis*	92	*Cheilotrema saturnum*	92	*Michthys muiiy*	91	N.A.	100	N.A.
A28	*Pogonias cromis*	91	*Cheilotrema saturnum*	92	*Michthys muiiy*	91	N.A.	100	N.A.
A29	*Pogonias cromis*	92	*Cheilotrema saturnum*	92	*Michthys muiiy*	92	N.A.	100	N.A.
B01	*Cynoscion microlepidotus*	100	*Atractoscion nobilis*	95	*Sciaenops ocellatus*	95	*Cynoscion microlepidotus*	100	*Cynoscion microlepidotus*
B02	*Cynoscion acoupa*	100	*Cynoscion parvipinnis*	96	*Cynoscion nobilis*	95	*Cynoscion acoupa*	100	*Cynoscion acoupa*
B03	*Cynoscion acoupa*	100	*Cynoscion parvipinnis*	96	*Cynoscion nobilis*	95	*Cynoscion acoupa*	100	*Cynoscion acoupa*
B11	*Cynoscion acoupa*	100	*Cynoscion parvipinnis*	96	*Cynoscion nobilis*	95	*Cynoscion acoupa*	100	*Cynoscion acoupa*
B13	*Cynoscion acoupa*	100	*Cynoscion parvipinnis*	96	*Cynoscion nobilis*	95	*Cynoscion acoupa*	100	*Cynoscion acoupa*
B17	*Cynoscion acoupa*	100	*Cynoscion parvipinnis*	96	*Cynoscion nobilis*	95	*Cynoscion acoupa*	100	*Cynoscion acoupa*
B20	*Cynoscion acoupa*	100	*Cynoscion parvipinnis*	96	*Cynoscion nobilis*	95	*Cynoscion acoupa*	100	*Cynoscion acoupa*
B21	*Cynoscion acoupa*	99	*Cynoscion parvipinnis*	96	*Cynoscion nobilis*	95	*Cynoscion acoupa*	100	*Cynoscion acoupa*
B22	*Cynoscion acoupa*	100	*Cynoscion parvipinnis*	96	*Cynoscion nobilis*	95	*Cynoscion acoupa*	100	*Cynoscion acoupa*
B23	*Cynoscion acoupa*	100	*Cynoscion parvipinnis*	96	*Cynoscion nobilis*	95	*Cynoscion acoupa*	100	*Cynoscion acoupa*
B25	*Cynoscion acoupa*	100	*Cynoscion parvipinnis*	96	*Cynoscion nobilis*	95	*Cynoscion acoupa*	100	*Cynoscion acoupa*
B26	*Cynoscion acoupa*	100	*Cynoscion parvipinnis*	96	*Cynoscion nobilis*	95	*Cynoscion acoupa*	100	*Cynoscion acoupa*
B27	*Cynoscion acoupa*	100	*Cynoscion parvipinnis*	96	*Cynoscion nobilis*	95	*Cynoscion acoupa*	100	*Cynoscion acoupa*
B28	*Cynoscion acoupa*	100	*Cynoscion parvipinnis*	96	*Cynoscion nobilis*	95	*Cynoscion acoupa*	100	*Cynoscion acoupa*
B32	*Cynoscion acoupa*	100	*Cynoscion parvipinnis*	96	*Cynoscion nobilis*	95	*Cynoscion acoupa*	100	*Cynoscion acoupa*
C05	*Otolithoides biaurithus*	99	*Atractoscion nobilis*	90	*Sciaenops ocellatus*	90	*Otolithoides biaurithus*	100	*Otolithoides biaurithus*
C07	*Otolithoides biaurithus*	99	*A. nobilis*	90	*Sciaenops ocellatus*	90	*Otolithoides biaurithus*	100	*Otolithoides biaurithus*
C08	*Otolithoides biaurithus*	99	*A. nobilis*	90	*Sciaenops ocellatus*	90	*Otolithoides biaurithus*	100	*Otolithoides biaurithus*
C12	*Otolithoides biaurithus*	99	*A. nobilis*	90	*Sciaenops ocellatus*	90	*Otolithoides biaurithus*	100	*Otolithoides biaurithus*
D01	*Lates niloticus*	100	*Lates calcarifer*	94	*Alepes kleinii*	92	*Lates niloticus*	77	*Lates niloticus*
D02	*Lates niloticus*	100	*Lates calcarifer*	94	*Alepes kleinii*	92	*Lates niloticus*	77	*Lates niloticus*
D03	*Lates niloticus*	100	*Lates calcarifer*	94	*Alepes kleinii*	92	*Lates niloticus*	77	*Lates niloticus*
D04	*Lates niloticus*	100	*Lates calcarifer*	95	*Alepes kleinii*	92	*Lates niloticus*	93	*Lates niloticus*

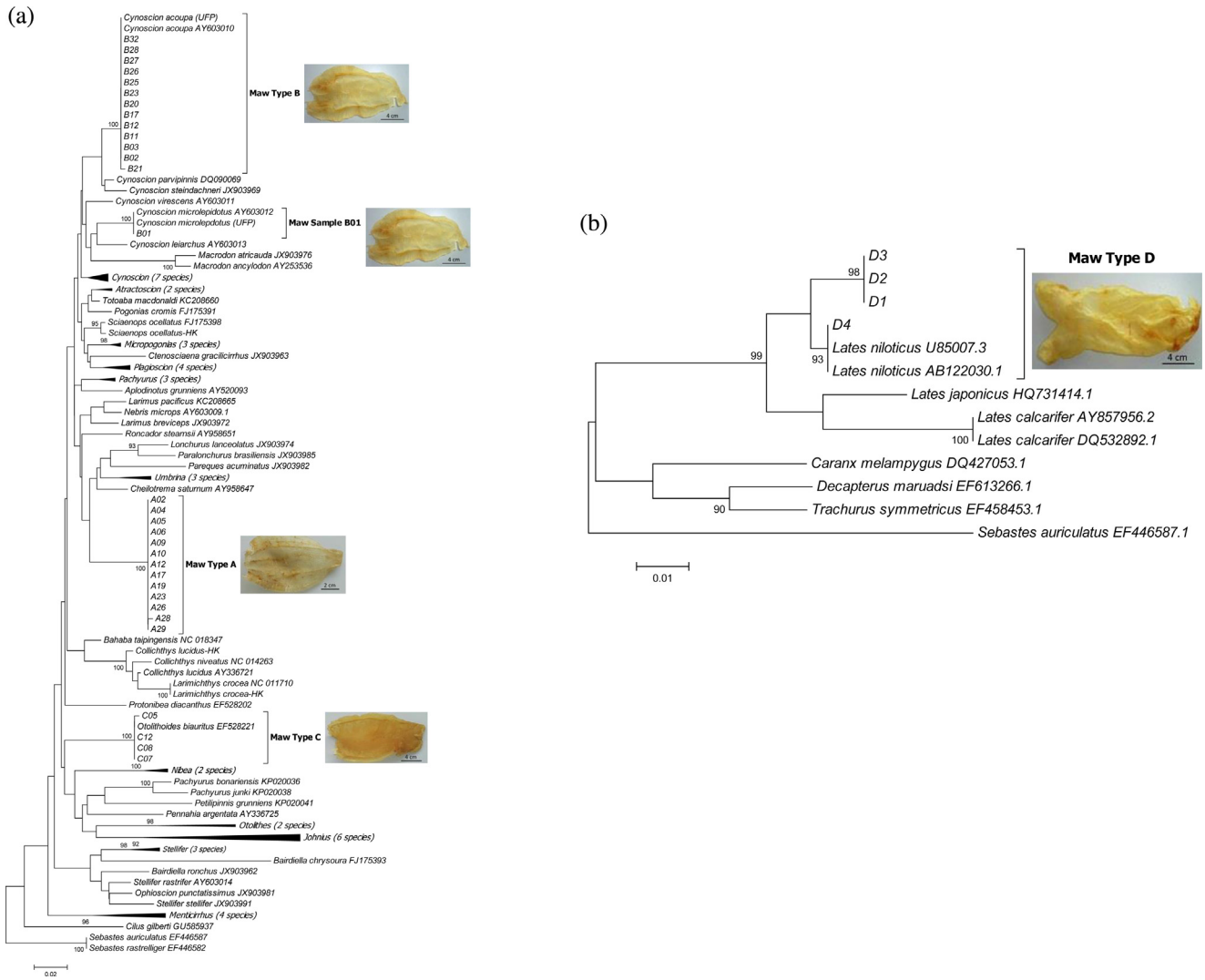


Fig. 3. Neighbour-joining tree of 16S region sequences for selected sequences from (a) Sciaenidae and (b) Latidae and 3 other families showing the placement of the fish maw samples. The significance of each branch is indicated by bootstrap test values (1000 replicates). Only values greater that 80% are shown for clarity. Maw specimens are coded as the maw type (A–D) followed by a number. Sequences ending with "UFP" represent reference sequences provided by the Federal University of Para, Brazil. Sequences ending with "HK" represent reference sequences obtained from fresh sample in Hong Kong.

Table 3
Kimura 2-parameter (K2P) divergence within and among clades using 16S rRNA sequences for fish maw specimens and reference database sequences. The maximum and minimum K2P pairwise sequence divergences are reported within each taxonomic group and the minimum K2P pairwise sequence divergences are reported between each taxonomic group and its nearest group.

Group	16S					Closest sister group
	Maw type	Number of samples	Within groups		Among groups	
			Max (%)	Min (%)	Min (%)	
1	A	13	0.5	0.0	9.0	N.A.
2	B (without sample B01)	14	0.2	0.0	2.9	*Cynoscion acoupa*
3	Sample B01	1	0.0	0.0	4.6	*Cynoscion microlepidotus*
4	C	5	0.5	0 .0	10.3	*Otolithoides biauritus*
5	D	4	1.3	0.0	4.8	*Lates niloticus*

barcoding accuracy (Meyer and Paulay, 2005). The partial sequence of the 16S rRNA gene employed in this study showed a generally low level of intraspecific divergence (0–1.6%) and a higher level of interspecific divergence (1.2–10.0%) for the sciaenids. The overlap of divergence was attributable to the lower divergence in some species pairs such as *Cynoscion arenarius* and *C. regalis*, *Micropogonias fumieri* and *M. undulatus*. The phylogenetic closeness of the former pair has also been reported in other molecular markers (Vergara-Chen et al., 2009).

In this study, the queried swim bladder specimens did not fall close to the taxa pairs of lower divergence. Moreover, all identified species formed clusters of very low within-group divergence and high bootstrap values, which gives high confidence in species identifications. The low overlap between intra- and interspecific divergence implies an accountable resolution of the 16S sequence among sciaenid species, which is also evidenced in studies on the same sequence in the same family (Díaz-Viloria et al., 2013; Lakra et al., 2009; Vinson et al., 2004). The relatively deep divergence

supported the discriminatory power of the 16S sequence for DNA barcoding in sciaenids (Lakra et al., 2009; Saitoh et al., 2009).

A limitation of species verification in this study was the incomplete coverage of database sequences for the family Sciaenidae. From the available sources, we obtained 16S sequences from 85 of the total ~280 species, with 41 of the total 66 genera of the family in the sequence database. The isolated cluster of the unidentified morphotype A suggests that it may belong to a genus not yet covered by the sequence database. Similarly insufficient coverage was observed for the family Latidae, to which the morphotype D was attributed. Reference sequences were available for only 3 of the 13 recognized species in the family. This low within-group divergence of 1.3% apparently represented a confident species assignment, but further confirmation is possible only with a more comprehensive sequence database. With the relatively robust species discrimination using the 16S sequence, continuous effort to expand species coverage is suggested to advance the resolution of the analyses. On the other hand, inclusion of other barcoding sequences, such as the rapidly developing COI sequence, would further enhance identification efficiency.

4.2. Difficulties in obtaining good quality DNA from processed/dried fish swim bladder

Failure to amplify the target sequence occurred in just over half of the fish swim bladder specimens. Multiple trials using different parts of individual bladders resulted in the same extraction/amplification success rates, suggesting a relatively uniform condition in different parts of the same swim bladder. The probable cause of the failure is degradation of DNA due to the curing process and/or poor preservation and storage. Storage of dried tissue can cause degradation of major DNA fragments to <100 bp in ~5 years and lead to amplification failure of ~500 bp barcoding sequence (Zimmermann et al., 2008). Dried swim bladder is typically prepared with long (i.e., decades) storage times in mind, according to Chinese tradition; in some cases older swim bladders are considered to be more valuable. Moreover, exposure to radiation in the sun-drying process is likely to accelerate DNA damage (Sinha and Hader, 2002). Bacterial contamination can also affect both amplification success and identification accuracy (Holmes et al., 2009). However, in the present study, all the successful amplicons were fish sequences, suggesting no bacterial contamination in the dried fish swim bladders; resistance to contamination is likely attributed to dehydration during processing. Higher success rates of DNA extraction have been noted for shark fins and it is not known why those for dried swim bladder are relatively low given apparent similarities in processing techniques. Unfortunately it was not possible to determine the details of swim bladder preparation to compare with those of shark fins.

4.3. Origin and value of the dried fish swim bladders in sampled Hong Kong retail outlets

Within the constraints of our current ability to resolve species identities based on DNA extractions discussed above, four croaker-like morphotypes were commonly sold as croaker swim bladders at the time of this study. Three of these, B, C, and D we confidently resolved to species or genus, at least one of which was not a croaker. Two of the four morphotypes, B and C, were identified by DNA as croakers. Swim bladders of type B were attributed to a combination of *Cynoscion acoupa* and *C. microlepidotus. Cynoscion* is one of the most speciose of sciaenid genera (Chao, 1978). *Cynoscion acoupa* is a high value commercial food fish that grows to a maximum of 110 cm total length (TL) and is distributed in the western Atlantic from Panama to Argentina. *C. microlepidotus* ranges from Venezuela to Santos, Brazil, in the western Atlantic, and grows to a maximum size of 92 cm TL. Both of these relatively large species are considered to be moderately to highly vulnerable to overfishing because of their high economic value and biological characteristics such as aggregation-spawning which can make them particularly easy to overfish (Cheung et al., 2005). *C. acoupa* is classified as near-threatened in Brazil which accounts for a large proportion of its geographic distribution (Chao et al., 2015). The maw from *Cynoscion* species in Brazil, Guyana and Venezuela are marketed as a value-added product (Labbish Chao, pers. comm.).

In the case of bladder type C, a single species, *Otolithoides biauritus*, is indicated. This species ranges from Pakistan, along the coasts of India and Sri Lanka, and southwards to the Malay Peninsula, Sumatra, Borneo and Vietnam (Lal Mohan, 1984). *O. biauritus* is found in coastal and inshore waters and grows to a maximum total length of 160 cm TL. It is an important food fish, the swim bladder of which is sold in both fresh and dried forms (Lal Mohan, 1984). Indeed, morphotype C was the most expensive of the collected swim bladders on sale with two factors probably playing a major role in its price; the proposed species by traders was *P. diacanthus*, valued for its rarity, and the bladder was thick, which, along with large size, is a highly considered characteristic (Lin, 1939). *P. diacanthus* (Indian name 'ghol') has been heavily targeted when available in recent years spurred on by the high export value of the swim bladder (e.g., Mudur 2012).

Morphotype D was identified as a freshwater species belonging to the family Latidae (perches). With the limited available sequences for this family, specimens of this morphotype best matched a single species, the Nile perch *Lates niloticus*. The species occurs in Africa, mainly throughout Ethiopia and in major river basins including the Nile, Chad, Senegal, Volta and Congo, and grows to a maximum body length of 200 cm TL (Ribbink, 1987). It inhabits channels, lakes and irrigation canals (Reed et al., 1967). It is an introduced species in many countries and there are reported adverse ecological impacts after its introduction (Ribbink, 1987). While *L. niloticus* is described as locally threatened in its native countries (Chad, Nile, Senegal, Congo and Volta) due to over harvesting, globally it is of least concern (Stone, 2007; IUCN 2015). Morphotype D, although not a croaker, was priced similarly to that of morphotype B, an average-priced croaker species. Interestingly, among the top countries indicated as exporters of fish maw to Hong Kong in the 2015 January to August CSD database are Tanzania and Uganda, both of which export the highly valued maw of the Nile perch to Asia. Such is the concern in Uganda about fishing pressure on the species (locally known as 'enuuni') resulting from the increasing value of the maw, that the government recently declared a ban on fishing immature individuals to protect the stock (Lekura, 2015).

Samples of morphotype A were clustered in Sciaenidae but could not be allocated to species because of a lack of match with any species available in the database. Given the low within-group divergence, it is likely that these specimens are all of one species in the family.

Traders reported that morphotype C was from India, and morphotype B from Peru. The origin of morphotype D was not known, while morphotype A was reported to be from local waters. From molecular analyses, morphotypes B and C are both Atlantic species, i.e. not occurring in India or Peru, while no morphotype was associated with any local species.

4.4. Conservation implications

It is not known what impact the trade in fish swim bladder has on species status or indeed how much it determines fishing activity on particular species. This is an important consideration given that several sciaenids are considered to be overfished and some are particularly threatened from fishing activity, including

on their spawning aggregations, when they may be particularly easy to overfish if unmanaged (Sadovy de Mitcheson et al., 2008; Tuuli, 2010). The blackspotted croaker, for example, common in the 1950s and 1960s in Hong Kong, has been heavily overfished in many areas, and all large croakers have disappeared from Hong Kong's once substantial fishery of this taxon (Tuuli, 2010). In the market survey relevant to this study, there were cases of dried swim bladders of morphotype A on sale, which were smaller and thinner, being reported by traders as the more expensive "Man Yue" (morphotype C, identified as *O. biauritus*). Both swim bladders are carrot-shaped making distinction by non-specialists difficult, but making it easy for traders to interchange or mistake the source species of swim bladders (Sasaki, 1989, 2001). Lack of species-specific fishery statistics in source countries for those fishes valued for their swim bladder, and poor information on their trade and provenance, severely limit the ability to trace the networks, value and volume of trade and hence to understand possible impacts on source stocks.

There is a particular interest in large and thick swim bladders which could account for the popularity of sciaenids and species with similar bladders, such as *L. niloticus*. It also means that there is more interest in larger species and these include species that are more readily overfished. Several are already threatened or near-threatened. Moreover, experience with the totoaba and Chinese bahaba, both listed as critically endangered, show that the rarer the swim bladder (for favoured forms) the more valuable it becomes. The Chinese bahaba is on the brink of extinction as a result of swim bladder trade and, in the extreme case of the totoaba, trade now appears to be largely for investment purposes (EIA, 2015).

In a twist of fate, the exploitation of the totoaba, which lives in a very limited geographic area in the Gulf of California, is also critically threatening the world's smallest porpoise and most endangered marine mammal, the vaquita (*Phocoena sinus*) (EIA, 2015). This species, which lives in the same area as the totoaba, is taken as bycatch with gillnets that target the totoaba. Despite protection, illegal fishing and trade in totoaba continues and the vaquita population numbers globally are critically low (Rojas-Bracho et al., 2006).

5. Conclusion

This study is the first to demonstrate the feasibility of DNA barcoding using the 16S rRNA sequence for species assignment for dried swim bladder on retail sale; it also highlighted several data gaps. The molecular approach is a valuable and viable tool for species identification in a trade that is clearly substantial but largely undocumented to species level, includes a number of high value and threatened species and in which species swim bladder interchangeability occurs due to the resemblance of differently valued swim bladders across species. However, this study was constrained by the relatively small number of croaker species in the available genetic reference databases. This is important given that large volumes of dried fish swim bladder from dynamic and diverse source species and countries are supplying the Chinese (including Hong Kong) markets. For example, recent information (Lorenzo Rojas-Bracho, pers. comm. 2014) suggested that a massive trade of swim bladder from gulf corvina (*Cynoscion othonopterus*) from Mexico to Southeast Asia may not be detected in the importing countries. The species is listed as vulnerable on the IUCN Red List and is heavily fished in its spawning aggregations with some management in place (Chao et al., 2010; Erisman et al., 2012).

In terms of monitoring, few croaker fisheries outside of the United States and Australia are monitored in any detail and import data into Hong Kong and mainland China lumped all dried swim bladder together with many other marine products (Clarke, 2002, 2004) until dried swim bladder were distinguished by the Harmonized Code System in January 2015. Moreover, as many people begin to eschew the use of shark fin in banquets, one of the replacement items being used is fish swim bladder so demand is expected to increase (In NH, 2013). Hence, overall, a better understanding of the trade dynamics, volumes, provenance and species involved in the provision of dried fish swim bladder would be extremely valuable and is now increasingly feasible using both molecular approaches and improved trade data monitoring.

Authors' contribution

C.D.T. performed sample collection, identification, laboratory work, analysis of the data and wrote part of the paper. Y.S. designed the study, and contributed to writing the biological and fisheries aspects of the manuscript. W.C.N. supervised and performed laboratory work and data analyses, and contributed to writing the molecular aspects of the manuscript.

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