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METHODS AND RESOURCES ARTICLE

First genetic evidence of illegal trade in endangered European eel (*Anguilla anguilla*) from Europe to Asia

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Abstract Eel farming in Asia relies on wild-caught juvenile "glass eels" of the genus *Anguilla*. When supplies of Japanese eels (*Anguilla japonica*) declined in the 1990s, Asian eel farming shifted to using European eels (*Anguilla anguilla*). The European eel is currently classified as "Critically Endangered", and export out of Europe has been suspended since March 2009. In early 2016, glass eels were seized at the Hong Kong International Airport and genetically identified using the COI barcode region. Samples matched *A. anguilla* with a similarity range of 99.39–99.85 %. To our knowledge, this is the first documented case of illegal trade of *A. anguilla* from Europe into Hong Kong using genetic evidence. Furthermore, multiple isolated incidents of eel seizures by customs indicate that Hong Kong is a major hub facilitating illegal trade in eels from Europe to Asia. We demonstrated that COI barcoding is a suitable tool in identifying illegally imported *A. anguilla*, which can support enforcement and prosecution as well as enable international cooperation between Europe and Asia.

Keywords Forensics · DNA barcoding · Wildlife trade · *Anguilla anguilla* · Hong Kong

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Introduction

The European eel (*Anguilla anguilla*) is among the most important commercial fish species in the world (Violi et al. 2015), and is currently classified as "Critically Endangered" by the International Union for Conservation of Nature (IUCN) Red List (Jacoby and Gollock 2014). In September 2007, *A. anguilla* was listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), which imposes regulation on its international trade. Since the listing came into effect on 13 March 2009, all Parties to the Convention are required to issue permits for all exports of the species (Jacoby and Gollock 2014). A range of reasons may account for the declines in *A. anguilla* stocks including migration barriers, changing oceanic conditions, predation, pollution, disease and parasites, and overexploitation (Jacoby et al. 2015). We postulate that the export of wild-caught "glass eel" (the transparent juvenile stage of the genus *Anguilla*) from Europe to Asia plays a significant role in the stock decline.

The glass eel trade is driven by demand for glass eels to supply eel farms in Asia. Given limited abundance of Japanese glass eels (*Anguilla japonica*) in the 1990s, European glass eels were increasingly used as a replacement (Crook 2010; Crook and Nakamura 2013). Decline in *A. anguilla* stocks caused export prices for European glass eels to increase significantly. In the 2000s, European glass

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eel was traded to Asia for more than 1000 €/kg (Anonymous 2007).

Since EU exports and imports of *A. anguilla* were suspended in December 2010 under Council Regulation (EC) No 338/97 (Anonymous 2014a), three strategies to circumvent conservation efforts have been documented: (1) Currently, juvenile *A. anguilla* are illegally traded from Europe to Asia for up to 1500 €/kg (Shiraishi and Crook 2015), (2) Eel exports from EU neighboring countries have increased (Crook and Nakamura 2013), and (3) trade in tropical *Anguilla* species has increased (Crook 2014; Nijman 2015). The yearly European demand for consumption and restocking is estimated to range from 27 to 35 tons (Anonymous 2014b). In contrast, 20 tons of *A. anguilla* glass eels were reportedly transferred into Chinese eel farms in 2013 and 2014 (Shiraishi and Crook 2015). Between 2009 and 2013, over 40 % of all eel products imported into Japan from mainland China were *A. anguilla* (Shiraishi and Crook 2015). Over the last 15 years, China and Japan have been the world's largest eel exporter and importer, respectively. (Shiraishi and Crook 2015).

The morphology of glass eels among *Anguilla* spp. is similar while only *A. anguilla* is CITES-listed. Difficulty in identifying the species masks the illegal trade, hinders law enforcement and also makes proof of such illegal trade challenging.

Materials and methods

Four items of luggage containing plastic bags filled with live glass eels, water and oxygen were seized at the Hong Kong International Airport on 05 January 2016. Bags were frozen and one bag from each luggage was delivered to the University of Hong Kong (HKU) for analysis. Specimens were sampled from each bag for analysis. Muscle tissues were incubated in 20 µL proteinase K at 56 °C overnight. DNA was extracted with a spin-column protocol described in DNeasy Blood and Tissue Kit (QIAGEN). A 655-bp fragment from the region of the mitochondrial COI gene was amplified using the primers FishF1 (TCA ACC AAC CAC AAA GAC ATT GGC AC) and FishR1 (TAG ACT TCT GGG TGG CCA AAG AAT CA) (Ward et al. 2005). PCR amplifications were performed in 20 µL volume including 14 µL of ddH2O, 2 µL 10× PCR buffer (Invitrogen^{TM}), 1.2 µL $MgCl_2$ (25 mM), 0.5 µL of each primer (10 µM), 0.4 µL dNTP, 0.2 µL BSA, 0.2 µL of *Taq* DNA polymerase (recombinant, Invitrogen^{TM}), and 1 µL of template DNA. Thermal cycling was performed at 94 °C for 2 min, followed by 35 cycles of 30 s at 94 °C, 40 s at 52 °C, and 72 °C at 1 min, with a final extension at 72 °C for 10 min. PCR products were visualized on a 1 % agarose gel. Amplicons were purified by incubating 5 µL of the PCR product with 2 µL of ExoSAP-IT reagent for 15 min at 37 °C followed by 15 min at 80 °C. The purified products were then submitted to the HKU Centre for Genomics Sciences for direct sequencing using the ABI 3730xl DNA Analyzer.

Paired-end sequences were assembled and aligned with Geneious 7.1.8. Identification of unknown sequences was conducted using BLAST to search the GenBank *nr* database, and the BOLD (Barcode of Life Data System) library. All sequences have been deposited in GenBank with accession number KU927484-89. Specimen data, sequences, trace files and primer details are available within the GEL project in BOLD (www.boldsystems.org). In addition, the publicly available COI sequences of *Anguilla* spp. were downloaded and compared with the sequences from our specimens. Sequence divergences were calculated using the Kimura two parameter (K2P) distance model (Kimura 1980). Phylogeny analysis was performed by MEGA v6.0 (Kumar et al. 1994). A neighbor-joining tree of K2P distances was constructed with 1000 bootstrap replicates.

Results

Six COI barcodes of 655-bp were obtained from a total of eight samples (two per bag), with at least one successful amplification from each bag. Searches were performed using the BOLD "Species Level Barcode Records" database, with the result that all specimens matched *Anguilla anguilla* with a similarity range of 99.39–99.85 % (Table 1). All records belong to the same Barcode Index Number (BIN), representing the species *A. anguilla* (BOLD:AAC6560).

Samples were then aligned with 160 sequences from the BOLD system for distance analysis. Contaminated or misidentified sequences were filtered from the analysis. The mean intra-species distance between public records of *A. anguilla* ($n = 63$) was 0.75 and the maximum intraspecific distance was 1.56 %. (Table 2). The nearest neighbor for *A. anguilla* was *A. rostrata* (GBGC0503-06), and the minimum interspecific distance and the nearest neighbor distance for *A. anguilla* were 2.60 and 2.43 % respectively. Individual BLAST searches with the GenBank resulted in matches with ≥99.8 % sequence identity (Table 1). In the neighbour-joining tree, all of our sequences clustered with *A. anguilla* with a maximum bootstrap support value of 100 % (Fig. 1).

To provide context for our genetic evidence, we collected information regarding the glass eel seizures from various sources including CITES authorities and media publications. For the years 2010–2015, we identified between one and seven seizures per year, conducted at

Table 1 Top match from BOLD (similarity percentage) and GenBank database (maximum identity percentage)

Sample number	Species identification	
	BOLD full database	GenBank (BLAST)
1	*Anguilla anguilla* (99.85 %)	*Anguilla anguilla* (99.8 %)
2.2	*Anguilla anguilla* (99.39 %)	*Anguilla anguilla* (100 %)
3.1	*Anguilla anguilla* (99.85 %)	*Anguilla anguilla* (99.8 %)
3.2	*Anguilla anguilla* (99.69 %)	*Anguilla anguilla* (99.7 %)
4.1	*Anguilla anguilla* (99.85 %)	*Anguilla anguilla* (100 %)
4.2	*Anguilla anguilla* (99.85 %)	*Anguilla anguilla* (99.8 %)

Table 2 Minimum and maximum intraspecific pairwise distances (%) for *Anguilla anguilla*, including mean and standard deviation (SD)

Species	Mean Intra-Sp	SD	Min Intra-Sp	Max Intra-Sp	Nearest neighbour	Nearest species	Distance to NN	Barcoding gap
A. anguilla	0.74	0.35	0.00	1.56	GBGC0503-06	*A. rostrata*	2.43	1.04

Nearest neighbour (NN) and the distance to NN was determined with the BOLD "Barcode Gap Analysis". Barcoding gap was calculated by the difference between maximum intraspecific and minimum interspecific genetic distances

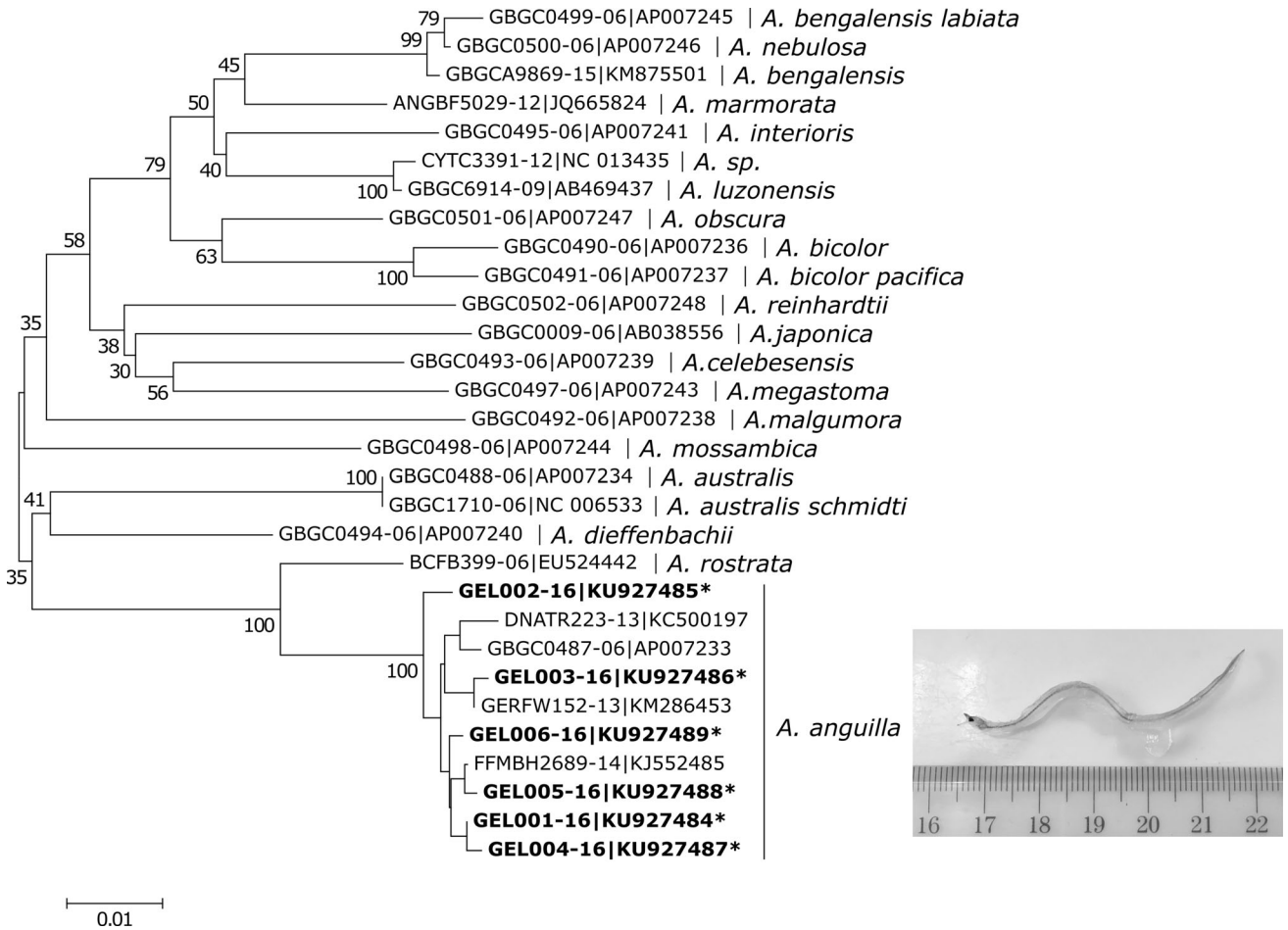

Fig. 1 Neighbour-joining (NJ) analysis of K2P-pairwaise distances from all 21 taxon identified from the BOLD public database. Taxon label denoted BOLD sample ID and GenBank Assession number respectively. *Numbers* represent bootstrap support for species clades for 1000 replicates. six samples from this study (*) are clustered within the clade *Anguilla anguilla. Scale* indicates 1 % distance. *Photo* displays a frozen specimen

international airports in Europe and Hong Kong. The transit route of four seizures went through Romania or Bulgaria. The European commission (EC) identified additional countries which have been or believed to be used as transit countries: Greece, Hungary, Albania, Former Yugoslav Republic of Macedonia, Morocco and Russia. Furthermore, the EC reports about several seizures, where large quantities of European glass eels where hidden in shipments of other fishery products or mislabeled (Anonymous 2016a). In 2016, 13 seizures were reported between 01 January and 08 March. All of them were destined for Hong Kong, except one, destined for Shanghai. Departure location of all seizures was Spain but the transit routes varied between Amsterdam, Paris, Madrid and Istanbul in Europe, and Dubai and Abu Dhabi in Saudi Arabia.

Discussion

Our DNA analyses clearly identified that *A. anguilla* has been illegally consigned from Madrid to Hong Kong in early January 2016. To our knowledge, this is the first time that the Europe-Asia trafficking route has been proven by genetic identification since export was suspended.

This study demonstrated the use of COI barcoding in identifying illegally traded *A. anguilla* glass eel from frozen seizure samples. The COI barcode region has emerged as a powerful molecular marker for species identification, and has been utilized for various wildlife forensics applications (Dawnay et al. 2007; Gonçalves et al. 2015; Asis et al. 2016). *A. anguilla* is a well-studied species with adequate reference sequence data. This data, taken together with a statement of confidence based on the phylogeny analysis, allowed us to unambiguously identify eel species as *A. anguilla*.

Our information on reported seizures indicate various smuggling routes to Asia but it is uncertain, which parameters determine the choice. Additionally, smuggling through Eastern Europe (Anonymous 2016a) might be applied in order to obscure the EU origin. We are careful in noting that glass eels which depart from Spain do not necessarily originate from Spanish glass eel fisheries. In Spain, live glass eel, which may be sourced from other European countries without tracking, can be legally sold for consumption. However, quantification and assessment of the seizure's temporal dynamic is not possible, since we were not able to gather reliable information from all relevant authorities. Hong Kong is known as a major wildlife trading hub for many species (Nijman 2010) and based on transit information from past seizures, European glass eels are no exception.

Since the EU trade ban came into effect, eel exports from non-EU countries to Asia have increased significantly (Crook 2014). On the one hand, these involve different *Anguilla* species such as *A. rostrata* from the Americas or Asia (e.g. tropical species *A. marmorata* and *A. bicolor*) (Crook 2014; Nijman 2015). On the other hand, countries neighboring the European Union which are located within the species native range, export European eels. It is uncertain if the eels of these exports originate from their territories, or if shipments include eels of European provenance. Future differentiation of EU-sourced eels from non-EU populations might be achieved by otolith microchemistry which has potential to determine the eels' provenance down to the estuary level (Reis-Santos et al. 2012; Evans et al. 2014). We postulate that the chemical signature of the last otolith increments will vary distinguishably according to the estuarine catchment environment and that these variations can be detected by multivariate statistics (Evans et al. 2014; Ziegler et al. 2016).

The genetic evidence of illegal trade of *A. anguilla* stressed an urgent need of stricter enforcement in both import and export countries. Although growing awareness and improved enforcement activity has resulted in an increased number of seizures in 2016 in Spain, we believe the illegal trade could be impeded by increasing transparency of trade activity and traceability of fished glass eel, as required by Council Regulation (EC) No. 1100/2007 (EU 2007) and intended by the "EU Action Plan against Wildlife Trafficking" (Anonymous 2016b). Therein, the European Commission aims to prioritize target species, such as eels, and improve cooperation among Member States and with non-EU countries, as well as key sources and transit countries.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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