Development and application of a novel real-time polymerase chain reaction assay to detect illegal trade of the European eel (Anguilla anguilla)

Diego Cardeñosa1,2 | Matthew J. Gollock3 | Demian D. Chapman4

1School of Marine and Atmospheric Science, Stony Brook University, Stony Brook, New York
2Fundación Colombia Azul, Bogotá, Colombia
3Conservation Programmes, Zoological Society of London, London, UK
4Department of Biological Sciences, Florida International University, Nebraska, Florida

Correspondence
Diego Cardeñosa, School of Marine and Atmospheric Science, Stony Brook University, Stony Brook, NY 11794. Email: diego.cardeosa@stonybrook.edu

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In 2007, the critically endangered European eel (Anguilla anguilla) was listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Flora and Fauna, due to declines in abundance across its range and significant international demand. Illegal trade in live European eel and its products is still occurring to satisfy the high demand around the world. Law enforcement officers face the challenge of identifying both live and processed European eel in a timely fashion to detain shipments and prosecute smugglers. Here, we describe the development of a reliable, field-based, fast, and cost-effective real-time polymerase chain reaction assay to detect European eels in trade. This novel assay was applied in a real law enforcement scenario, where positive results provided enough evidence to detain the shipment for more robust forensic analysis, leading to the first prosecution of eel smuggling in Hong Kong. Our approach could serve as a model for the development of other rapid and cost-effective tools to detect illegal wildlife trade where visual identification fails to provide enough evidence for prosecution. It could also enhance monitoring and enforcement of laws intended to protect highly traded and threatened species.

KEYWORDS
CITES, Hong Kong, law enforcement, qPCR, wildlife trade

1 | INTRODUCTION

The loss of biological diversity has become a prime global issue, but despite increasing interest and measures taken by the international community, rates of biological diversity loss are still increasing (Butchart et al., 2010). Unsustainable exploitation and associated wildlife trade, pollution, and climate change are among the most pressing global issues (Butchart et al., 2010; Diaz & Rosenberg, 2008; Hoegh Guldberg et al., 2007; Jones et al., 2013; Milliken & Shaw, 2012; Reeve, 2006; Schiller, Bailey, Jacquet, & Sala, 2018), and several multilateral environmental agreements have been created as part of the international legal framework to manage biodiversity.

The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) came into force in 1975 to regulate the international trade of threatened species, holding the 183 signatory Parties (as of March 2019) accountable when trade occurs without adhering to the terms of the treaty. Species can be listed in one of three appendices: international trade in Appendix I species is prohibited unless authorized in exceptional circumstances; Appendix
II-listed species must be exported and imported such that trade is sustainable, traceable, and legal; and Appendix III regulates the trade of a species at the request of a specific Party requiring international co-operation (https://www.cites.org/eng/app/index.php). Since its creation, CITES Parties have listed over 35,000 plant and animal species in its appendices, with the large majority (96%) of these species listed in Appendix II (CITES, 2012). International trade of CITES Appendix II listed species the issuance of permits certifying that specimens were legally caught, export is not detrimental to the survival of the species, and traded specimens are traceable through the supply chain (Vincent, Sadovy de Mitcheson, Fowler, & Lieberman, 2014). Importing parties are required to inspect wildlife shipments and make reasonable efforts to interdict illegal trade (i.e., trade in CITES listed species without the correct permits).

The European eel (Anguilla anguilla) has a complex catadromous life cycle, where maturing individuals—“silver eels”—leave continental waters ranging from Northern Norway to North Africa and the Mediterranean, migrate to their spawning grounds, at present believed to be the Sargasso Sea, before they breed and die (Tesch, 1978; van Ginneken & Maes, 2005). After hatching, larvae, referred to as leptocephali, are then transported by ocean currents back to the eastern Atlantic during which time they elongate, and on arrival to estuaries and coastal waters, become what are commonly referred to as “glass eels” (Tesch, 2003). Once established in continental waters, they feed and grow as “yellow eels” before transforming to silver eels in readiness for the spawning migration (Tesch, 2003). The generation length of the European eel is presently believed to be 15 years on average (Jacoby & Gollock, 2014). Before the 1990s, European glass eels were primarily captured and farmed in Europe to supply local demand (Ringuet, Muto, & Raymakers, 2002). Globally, farming is responsible for 90% of anguillid eel production (Crook, 2010), because breeding in captivity is not presently commercially viable (Shiraishi & Crook, 2015). There has been increasing concern for the status of the European eel over the past few decades, and there are a range of threats, including unsustainable exploitation and trade, that are believed to have impacted the stock (ICES, 2018). Indeed, A. anguilla is presently listed “critically endangered” by the International Union for Conservation of Nature Red List of Endangered Species (Jacoby & Gollock, 2014). A number of conservation and management measures have been implemented in the past two decades, including the creation of Council Regulation (EC) No 1100/2007 for “establishing measures for the recovery of the stock of European eel” and the listing of A. anguilla in Appendix II of CITES. Owing to the species’ status, the EU’s Scientific Review Group concluded that it was not possible to perform a non-detriment finding—the assessment that trade does not threaten an Appendix II listed species—for the export of A. anguilla in December 2010 (EC, 2010), and a zero-import/export quota was set which remains in place at the time of writing (EC, 2018).

Illegal international trade of live and processed European eel is challenging to detect due to the very limited visual identification characteristics for differentiating from other commonly traded anguillid eel species, (i.e., the Japanese eel [Anguilla japonica], the American eel [Anguilla rostrata], the giant mottled eel [Anguilla marmorata], and the Indonesian short-fin eel [Anguilla bicolor], all of which can still legally be traded internationally (Musing et al., 2018). There is growing evidence that European eels are illegally exported from the EU to East Asia (Musing et al., 2018; Stein et al., 2016), and one of the major problems for law enforcement officers is the inability to identify the species of traded live eels or meat, in a timely manner (Musing et al., 2018). While commonly used wildlife forensics tools (e.g., DNA barcoding) have proven useful to detect illegal wildlife trade (Cardeñosa et al., 2018; Gonçalves, Oliveira-Marques, Matsumoto, & Miyaki, 2015; Stein et al., 2016), most of these tools require transferring tissue samples to a laboratory away from the port-of-entry and going through complex process of DNA barcoding, which can take hours or days. A more portable, faster, and potentially more cost-effective approach for detection of illegal wildlife trade that can be deployed at the port-of-entry is real-time polymerase chain reaction (rtPCR). This tool requires the use of a molecular dye (e.g., SYBR Green) and species-specific primers to detect a particular target species or group of species if primers are multiplexed (Cardeñosa, Quinlan, Shea, & Chapman, 2018). Here, we present a rapid (~2 hr), mobile, and cost-effective (<$1.00 USD per sample) real-time PCR assay to detect the illegal trade of A. anguilla, using meat, glass eels, or water used for transportation as the source of DNA. This protocol can provide sufficient information to authorities around the world to hold the shipments and help prosecute offenders based on the presence of CITES listed A. anguilla. We also present a case-study conducted in Hong Kong that allowed authorities to detect the smuggling of illegally-traded live glass eels of A. anguilla at the Chek Lap Kok Hong Kong International Airport.

2 METHODOLOGY

Previously deposited mitochondrial genomes for A. anguilla and A. rostrata were downloaded from Genbank (Data S1) and aligned in Seaview v4 (Gouy, Guindon, & Gascuel, 2010). Species-specific primers EU_Eel_F (forward primer 5’-AATAAGAGATCACCAAYCAAATAAATC-3′) and EU_Eel_R (reverse primer 5’-GGCTTGGTGAGTAACG
AGTCTAATGTA-3') were designed based on nucleotide differences between the target *A. anguilla* sequences and the non-target and closely related *A. rostrata* (Figure 1).

DNA extractions of previously seized samples of *A. anguilla* and *A. rostrata*, provided by the Agriculture, Fisheries and Conservation Department (AFCD) of Hong Kong, were conducted using previously published Chelex DNA extraction protocols (Cardeñosa, Hyde, & Caballero, 2014). Briefly, 200 μL of 10% Chelex solution were added to a PCR tube with a small tissue sample (~2mm²) and heated in the QuantStudio5 System (Thermofisher) for 20 min at 60°C and for 25 min at 99°C, followed by a brief centrifugation and storage at 4°C. A no-template negative control was added to check for reagent contamination. To test the most stringent annealing temperature for the designed primers, each 20 μL reaction included, 10 μL of PowerUp SYBR Green Master Mix (Applied Biosystems), 2.5 μL from 10 μM stock solutions of each primer, and 5 μL of extracted DNA. Designed primers were tested against a wide range of annealing temperatures (50–65°C) on the QuantStudio5 System (Thermofisher), and 60°C was selected as the most stringent annealing temperature to be used in further trials to amplify the target species optimally. The thermal PCR cycling profile was as follows: 50°C for 2 min, 95°C for 10 min, followed by 30 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 45 s. The primers were also tested for full specificity against 10 glass eel samples of the target species *A. anguilla*, 10 glass eel samples of *A. rostrata*, and five meat samples of each of the remaining anguillid eels common in the international trade: *A. japonica*, *A. marmorata*, and *A. bicolor*.

Once the full specificity of the protocol was validated, it was tested in a case-study in collaboration with AFCD inspectors in Hong Kong. During this field test, the QuantStudio5 System (Thermofisher), reagents, and plastic consumables were transported to the Chek Lap Kok Hong Kong International Airport, where a suspected case of smuggling live glass eels of *A. anguilla* was taking place. Four suitcases, each containing 10 plastic bags with live glass eels were seized by the Hong Kong authorities. One plastic bag from each suitcase was randomly selected, and 20 individual eels were randomly chosen to extract the DNA and run the protocol as described above. In addition, 1.8 mL of the water, from two randomly selected bags from each suit case (n = 8), were taken in individual Eppendorf tubes and stored at 4°C for further analysis.

Chelex resin (0.2 g) was added and mixed to each Eppendorf tube, and 200 μL were taken and added to individual PCR tubes to be heated in the QuantStudio5 System (Thermofisher) following the Chelex DNA extraction protocol described above. Next, 5 μL of the extracted DNA were added to a PCR tube with 10 μL of PowerUp SYBR Green Master Mix (Applied Biosystems) and 2.5 μL from 10 μM stock solutions of each primer and run in the QuantStudio5 System (Thermofisher) with the thermal profile described above to assess the detection of *A. anguilla* from sampled eels and water they were contained in.

### 3 RESULTS

The putative species-specific primers for *A. anguilla* performed optimally at an annealing temperature of 60°C and providing a highly reliable detection of *A. anguilla* with 0% false negatives in our testing (Figure 2). The assay failed to amplify any of the non-target species under these conditions (i.e., there were no false positives; Figure 2). The protocol also achieved the identification of live glass eels of *A. anguilla* during a smuggling case at the Chek Lap Kok Hong Kong International Airport (Figure 3a). Our rtPCR assay positively identified 100% of the samples tested during the case-study as originating from this species. Notably, the identification of *A. anguilla* was subsequently confirmed by posterior DNA sequencing of the samples with no presence of false positives or false negatives (AFCD pers. comm.). Additionally, we were able to identify the presence of *A. anguilla* in all water samples taken from the bags at the Chek Lap Kok Hong Kong International Airport (Figure 3b).

### 4 DISCUSSION

The protocol described here is a rapid, cost-effective and reliable tool to detect the CITES Appendix II listed...
A. anguilla in trade—this in turn, depending on the export nation, can determine whether the species is being traded illegally or not. The protocol was able to achieve full specificity, failing to amplify the other Anguilla spp. commonly found in international trade. We were unable to test the designed primers on DNA samples from all described Anguilla species (n = 15) and no sequences were found for the used locus from the remaining 10 species in the National Center of Biotechnology Information (NCBI) GeneBank (http://www.ncbi.nlm.nih.gov/genbank/) to check for primer

**FIGURE 2** Amplification plot showing positive amplification of the CITES-listed *Anguilla anguilla* and negative amplifications of all non-target species tested. CITES, Convention on International Trade in Endangered Species of Wild Flora and Fauna

**FIGURE 3** Amplification plots for the field-based test showing the detection of the CITES-listed *Anguilla anguilla*. (a) The amplification plot of the glass eels tested, and (b) the amplification plot of water samples taken from the seized bags containing live *A. anguilla*. CITES, Convention on International Trade in Endangered Species of Wild Flora and Fauna
mismatches. Nevertheless, it is unlikely that any of the untested Anguilla species would amplify, based on (a) the number of mismatches found in the forward primer region between A. anguilla and its phylogenetically closest relative A. rostrata (Figure 1), and (b) the complete specificity of the designed primer against the remaining tested species. The protocol also proved to be highly efficient, amplifying 100% of the tested glass eel samples during the smuggling case-study at the Chek Lap Kok Hong Kong International Airport of Hong Kong (Figure 3a). Moreover, the protocol was able to detect the presence of A. anguilla in 100% of the water samples taken from the plastic bags they were contained in (Figure 3b). Presumably amplifying environmental DNA (eDNA) from excreta, sloughing, and dead specimens, this provides a novel non-invasive means to rapidly screen large shipments of glass eels for the presence of A. anguilla, which reduces the risk of spoiling/damage that can deter detainment (Musing et al., 2018). Overall, our testing suggests that positive result from this protocol from glass eels, meat, or water containing glass eels constitutes robust evidence of the presence of A. anguilla, providing probable cause for the authorities to detain a shipment and provide them the time and justification for the cost of conducting a more detailed, and more widely accepted evidentiary genetic investigation (e.g., DNA barcoding under controlled laboratory conditions).

Other genetic identification methods have been developed in the past to detect commonly found Anguilla species in raw and processed products using restriction fragment length polymorphisms (RFLPs; Rehbein et al., 2014), allelic discrimination techniques (Tanaka et al., 2014), and even rtPCR techniques similar to the one described in this study (Trautner, 2013). Trautner (2013) describes an rtPCR protocol to differentiate A. anguilla, A. rostrata, and A. japonica for restocking purposes with a similar methodology, but with key differences for law enforcements scenarios. The main differences are that our species-specific primer has been validated across all Anguilla species commonly found in the international trade, a new sampling methodology is described (i.e., water sampling described above), and our protocol has been applied to real law enforcement cases, leading to the first prosecution of eel smuggling in Hong Kong, where authorities are now fully equipped to used real-time PCR techniques to detect illegal trade of CITES-listed eels (this study) and sharks (Cardeñosa, Quinlan, et al., 2018). It has been recognized that illegal trade is well organized and uses multiple, shifting routes (Musing et al., 2018); therefore, it is possible that this screening capacity will cause further shifts in illegal behaviors and as such it is essential that international collaboration is strengthened and use of techniques, such as the one described here, are used widely.

Future studies should focus on validating Trautner (2013) primers for A. rostrata and A. japonica across all Anguilla species, and design new species-specific primers for the rest of the tropical Anguilla species, to monitor the increasing international trade and improve traceability of their products (Crook, 2014; Crook & Nakamura, 2013; Gollock, Shiraishi, Carizzo, Crook, & Levy, 2018).

The protocol described here for A. anguilla and the recently described protocol for shark CITES-listed species (Cardeñosa, Quinlan, et al., 2018) represent the most rapid and least expensive molecular identification protocols to detect illegal trade in CITES-listed species to our knowledge. These techniques can be customized to most, if not all, wildlife trade products where DNA can be extracted and species-specific primers can be designed. These rapid and cost-effective tools to resolve species identification, and detect illegal wildlife trade, should be developed for all traded wildlife products where visual identification fails to provide enough evidence for prosecution. The development of future molecular identification tools should (a) provide reliable identification with no false-positives, and (b) fit the needs, budget, and time constrains for its intended users (e.g., authorities in developing countries). The development of tools and protocols should also be accompanied by capacity building and international cooperation between researchers, managers, and industry, both within and between developed and developing countries. This sharing of expertise makes the most of available resources and optimizes implementation. Wildlife crime has become a major global issue and is a primary driver of biodiversity loss, as such, multiple tools are required for the implementation and enforcement of international laws intended to protect threatened species.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

D.C. and D.D.C conceived the experiment. D.D.C and D.C. obtained funding. D.C. conducted the analysis in the
lab and analyzed the results. MG provided samples of *Anguilla bicolor* and *Anguilla marmorata*. D.C. conducted the field test. D.C. and D.D.C. wrote the manuscript. All authors provided comments and edits to the manuscript.

**DATA ACCESSIBILITY**

For the development of this protocol no database was created. All data is presented in the manuscript.

**ETHICS STATEMENT**

Ethical clearances were not required for the development of this protocol.

**ORCID**

Diego Cardeñosa [https://orcid.org/0000-0001-9414-7385](https://orcid.org/0000-0001-9414-7385)

**REFERENCES**

Butchart, S. H. M., Walpole, M., Collen, B., van Strien, A., Scharlemann, J. P. W., Almond, R. E. A., … Watson, R. (2010). Global biodiversity: Indicators of recent declines. *Science*, 328, 1164–1168.

Cardeñosa, D., Fields, A. T., Babcock, E., Zhang, H., Feldheim, K., Shea, S. K. H., … Chapman, D. D. (2018). CITES-listed sharks remain among the top species in the contemporary fin trade. *Conservation Letters*, 43, e12457–e12457.

Cardeñosa, D., Hyde, J., & Caballero, S. (2014). Genetic diversity and population structure of the pelagic thresher shark (*Alopias pelagicus*) in the Pacific Ocean: Evidence for two evolutionarily significant units. *PLoS One*, 9, e110193–e110112.

Cardeñosa, D., Quinlan, J., Shea, K. H., & Chapman, D. D. (2018). Multiplex real-time PCR assay to detect illegal trade of CITES-listed shark species. *Scientific Reports*, 8, 1–10.

CITES. 2012. Appendices I, II and III: valid from September 25, 2012. Retrieved from: http://www.cites.org/eng/app/appendices.php

Crook, V. (2010). Trade in Anguilla species, with a focus on recent trade in European eel *A. Anguilla*. TRAFFIC report prepared for the European Commission, 1–56.

Crook, V. (2014). Slipping away: International *Anguilla* eel trade and the role of the Philippines. *TRAFFIC and ZSL*, UK, 1–54.

Crook, V., & Nakamura, M. (2013). Glass eels: Assessing supply chain and market impacts of a CITES listing on Anguilla species. *TRAFFIC Bulletin*, 25, 24–30.

Diaz, R. J., & Rosenberg, R. (2008). Spreading dead zones and consequences for marine ecosystems. *Science*, 321, 926–929.

EC. (2010). Short summary of conclusions of the 54th meeting of the Scientific Review Group on Trade in Wild Fauna and Flora, 1–3.

EC. (2018). Short summary of conclusions of the 85th meeting of the Scientific Review Group on trade in wild fauna and flora, Brussels, 1–4.

Gollock, M., Shiraishi, H., Carrizo, S., Crook, V., Levy, E. (2018). Status of non-CITES listed anguillid eels. AC30 Doc. 18.1, Annex 2, 1–176.

Gonçalves, P. F. M., Oliveira-Marques, A. R., Matsumoto, T. E., & Miyaki, C. Y. (2015). DNA barcoding identifies illegal parrot trade. *The Journal of Heredity*, 106(Suppl 1), 560–564.

Gouy, M., Guindon, S., & Gascuel, O. (2010). SeaView version 4: A multiplatform graphical user Interface for sequence alignment and phylogenetic tree building. *Molecular Biology and Evolution*, 27, 221–224.

Hoegh Guldberg, O., Mumby, P. J., Hooten, A. J., Steneck, R. S., Greenfield, P., Gomez, E., … Hatziolos, M. E. (2007). Coral reefs under rapid climate change and ocean acidification. *Science*, 318, 1737–1742. https://doi.org/10.1126/science.1152509

ICES. 2018. Report of the Joint EIFAAAC/ICES/GFCM Working Group on Eels (WGEE). Gdańsk, Poland. ICES CM ACOM, 1–151.

Jacoby, D. M. P., Gollock, M.J.. (2014). *Anguilla anguilla*. The IUCN red list of threatened species. Version 2014.3. Retrieved from www.iucnredlist.org

Jones, D. O. B., Yool, A., Wei, C.-L., Henson, S. A., Ruhl, H. A., Watson, R. A., & Gehlen, M. (2013). Global reductions in seafloor biomass in response to climate change. *Global Change Biology*, 20, 1861–1872.

Milliken, T., & Shaw, J. (2012). The South Africa–Vietnam rhino horn trade nexus: A deadly combination of institutional lapses, corrupt wildlife industry professionals and Asian crime syndicates. Johannesburg, South Africa: TRAFFIC.

Musing, L., Shiraishi, H., Crook, V., Gollock, M., Levy, E., Kececnagy, K. (2018). Implementation of the CITES appendix II listing of European eel *Anguilla Anguilla*, 1, 1–82.

Reeve, R. (2006). Wildlife trade, sanctions and compliance: Lessons from the CITES regime. *International Affairs*, 82, 881–897.

Rehbein, H., Sotelo, C. G., Perez-Martin, R. I., Chapela-Garrido, M. J., Hold, G. L., Russell, V. J., … Rey-Mendez, M. (2014). Differentiation of raw or processed eel by PCR-based techniques: Restriction fragment length polymorphism analysis (RFLP) and single strand conformation polymorphism analysis (SSCP). *European Food Research and Technology*, 214, 171–177. https://doi.org/10.1007/s00217-001-0457-y

Ringuet, S., Muto, F., & Raymakers, C. (2002). Eels: Their harvest and trade in Europe and Asia. *TRAFFIC Bulletin*, 19, 1–26.

Schiller, L., Bailey, M., Jacquet, J., & Sala, E. (2018). High seas fisheries play a negligible role in addressing global food security. *Science Advances*, 4, eaat8351.

Shiraishi, H., & Crook, V. (2015). Eel market dynamics: An analysis of *Anguilla* production, trade and consumption in East Asia (pp. 1–47). Tokyo, Japan: TRAFFIC.

Stein, F. M., Wong, J. C. Y., Sheng, V., Law, C. S. W., Schröder, B., & Baker, D. M. (2016). First genetic evidence of illegal trade in endangered European eel (*Anguilla Anguilla*) from Europe to Asia. *Conservation Genetics Resources*, 8, 1–5.

Tanaka, C., Shirotori, F., Sato, M., Ishikawa, M., Shinoda, A., Aoyama, J., & Yoshinaga, T. (2014). Genetic identification method for two subspecies of the Indonesian short-finned eel, *Anguilla bicolor*, using an allelic discrimination technique. *Zoological Studies*, 53, 1–7.
Tesch, F.-W. (1978). Telemetric observations on the spawning migration of the eel (*Anguilla Anguilla*) west of the European continental shelf. *Environmental Biology of Fishes*, 3, 203–209.

Tesch, F.-W. (2003). In J. E. Thorpe (Ed.), White RJ, translator *The Eel* (3rd ed., pp. 1–418). Oxford, UK: Blackwell Publishing Company.

Trautner, J. H. (2013). Stocking the right eel species: A fast PCR-based identification assay to discriminate European (*Anguilla Anguilla* (Linnaeus, 1758)), American (*A. rostrata* (Lesueur, 1817)) and Japanese eel (*A. japonica* (Temminck & Schlegel, 1846)). *Journal of Applied Ichthyology*, 29, 912–915.

van Ginneken, V. J. T., & Maes, G. E. (2005). The European eel (*Anguilla Anguilla*, Linnaeus), its lifecycle, evolution and reproduction: A literature review. *Reviews in Fish Biology and Fisheries*, 15, 367–398.

Vincent, A. C. J., Sadovy de Mitcheson, Y. J., Fowler, S. L., & Lieberman, S. (2014). The role of CITES in the conservation of marine fishes subject to international trade. *Fish and Fisheries*, 15, 563–592.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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