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DNA Barcoding Identifies Illegal Parrot Trade

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Abstract

Illegal trade threatens the survival of many wild species, and molecular forensics can shed light on various questions raised during the investigation of cases of illegal trade. Among these questions is the identity of the species involved. Here we report a case of a man who was caught in a Brazilian airport trying to travel with 58 avian eggs. He claimed they were quail eggs, but authorities suspected they were from parrots. The embryos never hatched and it was not possible to identify them based on morphology. As 29% of parrot species are endangered, the identity of the species involved was important to establish a stronger criminal case. Thus, we identified the embryos' species based on the analyses of mitochondrial DNA sequences (cytochrome c oxidase subunit I gene [COI] and 16S ribosomal DNA). Embryonic COI sequences were compared with those deposited in BOLD (The Barcode of Life Data System) while their 16S sequences were compared with GenBank sequences. Clustering analysis based on neighbor-joining was also performed using parrot COI and 16S sequences deposited in BOLD and GenBank. The results, based on both genes, indicated that 57 embryos were parrots (*Alipiopsitta xanthops*, *Ara ararauna*, and the [*Amazona aestiva*/*A. ochrocephala*] complex), and 1 was an owl. This kind of data can help criminal investigations and to design species-specific anti-poaching strategies, and demonstrate how DNA sequence analysis in the identification of bird species is a powerful conservation tool.

Resumen

El trafico ilegal amenaza la supervivencia de muchas especies silvestres, y la ciencia forense molecular puede esclarecer diversas preguntas planteadas durante la investigación de casos de comercio ilegal. Entre estas preguntas está la identidad de las especies implicadas. Aquí se presenta el caso de un hombre que fue sorprendido en un aeropuerto brasileño intentando viajar con 58 huevos de aves. Él afirmó que eran huevos de codorniz, pero las autoridades sospecharon que eran huevos de loro. Los embriones nunca eclosionaron y no fue posible identificarlos con base en su morfología. Como el 29% de las especies de loros están en peligro, detectar la identidad de las especies involucradas era importante para establecer un caso criminal más fuerte. Así, se identificaron las especies de los embriones con base en el análisis de secuencias de ADN mitocondrial (COI y ADN ribosomal 16S). Las secuencias de COI de los embriones se compararon con las depositadas en BOLD (*The Barcode of Life Data System*), mientras que las secuencias de 16S fueron comparadas con secuencias de GenBank. También se llevó a cabo el análisis de agrupamiento basado en *neighbor-joining*, utilizando todas las secuencias de COI de loro depositadas en BOLD. Los resultados, basados en ambos

genes, indicaron que 57 embriones eran loros (*Alipiopsitta xanthops, Ara ararauna*, y el complejo [*Amazona aestiva/A. ochrocephala*]), y uno era un búho. Estos tipos de datos pueden ser utilizados para construir un caso legal más fuerte, para diseñar estrategias de lucha contra la caza furtiva de especies específicas y demostrar cómo el análisis de secuencias de ADN para la identificación de especies de aves es una herramienta poderosa de conservación.

Subject area: Conservation genetics and biodiversity
Key words: COI, DNA barcoding, embryos, Psittaciformes, 16S rDNA, wildlife illegal traffic

Introduction

Illegal wildlife trade can impact the survival of species by the introduction of pathogens and invasive species and by overexploitation (reviewed in Rosen and Smith 2010). Molecular markers have been applied in various illegal trade cases and are especially useful in analyses of forensic samples that cannot be morphologically identified. For example, DNA sequences were used to identify species of whales from Japanese markets (Baker and Palumbi 1994), as well as the geographic origin and sex of leopard skins in India (Mondol et al. 2014). Here we report a case from 2003 of a man who was arrested at Recife International Airport in Brazil carrying 58 unhatched avian eggs and intending to fly to Europe. The eggs were packed around his abdomen to keep them alive during the trip. The police were already investigating him, and during this trip to Brazil he visited various states where he could have acquired the eggs. When arrested, he claimed that they were quail eggs, but their external morphology did not support his claim. The embryos never hatched; thus, it was not possible to identify them based on their morphology. However, the external morphology of the eggs and embryos suggested that they were from parrots. These birds are commonly kept as pets and the illegal capture of individuals in the wild for the pet trade threatens many parrot species (Guedes and Harper 1995).

There are 83 parrot species in Brazil (Clements et al. 2013), and 21 are considered endangered at some level (BirdLife International 2014). Based on surveillance of this man's travel within Brazil, these eggs could have belonged to any of these 83 species. According to Brazilian law, the use of wildlife without permission, license, or authorization from the competent authority can result in detention and penalty (nº 9.605/98 – Article 29). The penalty increases if the crime is committed against rare or endangered species. Thus, the identity of the eggs' corresponding species was important to establish a potentially stronger case.

Mitochondrial gene sequences can be useful in species identification (Avise 2004; Armstrong and Ball 2005; Markmann and Tautz 2005; Monaghan et al. 2005; Vences et al. 2005a; Roe and Sperling 2007; Smith et al. 2008a, 2008b). A landmark in molecular species identification was the adoption of a 648 base pair (bp) fragment of the mitochondrial cytochrome c oxidase subunit I gene (COI) as a standard marker to identify (barcode) all species (Hebert et al. 2003a). The ideal sequence database should have COI sequences (DNA barcodes) from identified specimens of all described species deposited in curated collections. This database could then be used for comparison with a COI sequence from an unidentified organism. However, Vences et al. (2005b) recommended the use of another mitochondrial gene with more conserved priming sites, the 16S ribosomal DNA gene (16S rDNA), as an additional standard DNA barcoding marker for vertebrates. Despite criticisms (Moritz and Cicero 2004; Rubinoff 2006), DNA barcoding has been successfully used for species identification in a variety of animal taxa (Hebert et al. 2003a, 2003b, 2004; Kerr et al. 2007; Ward et al. 2005; Tavares et al. 2011). Furthermore, it has allowed the identification of species from forensic wildlife samples (e.g. Dawnay et al. 2007; Dalton and Kotze 2011).

Because the species of these 58 unhatched embryos could not be identified morphologically, the aim of the present study was to identify the species based on mitochondrial COI and 16S rDNA sequences. Even though these markers are linked, this comparison allowed us to ask whether differential taxonomic representation in 2 databases (The Barcode of Life Data System [BOLD] and GenBank) could influence results. This approach was successfully applied in a previous study using molecular markers to identify embryos of avian species from eggs apprehended in Australia (Coghlan et al. 2012).

Materials and Methods

Tissue samples from the 58 apprehended embryos were preserved in 100% ethanol at –20 °C and were deposited in the collection of Laboratório de Genética e Evolução Molecular de Aves, Instituto de Biociências, Universidade de São Paulo. Total DNA was extracted using a standard proteinase K/phenol-chloroform protocol (Bruford et al. 1992).

A fragment of COI was amplified using the primer pairs LTyr (5′ TGTAAAAAGGWCTACAGCCTAACGC 3′; Tavares and Baker 2008) and COIH7557 (5′ GGCGGATGTGAAGTATGCTCGGG 3′; Tavares and Baker 2008). When polymerase chain reaction (PCR) failed and degraded DNA was the suspected cause, primer BirdR1 (5′ ACGTGGGAGATAATTCCAAATCCTG 3′; Hebert et al. 2004) was used with LTyr. A fragment of the 16S rDNA was amplified by PCR using primers 16SH3309 (5′ TGCGCTACCTTCGCACGGT 3′; Tavares et al. 2004) or 16SH3024 (5′ TTACTCATTTTAGCATTRGTTCA 3′; RibasCC, personal communication), and 16SL2702 (5′ CCTACCGAGCTGGGTGATAGCTGGTT 3′; Miyaki et al. 1998). PCR was performed in a total volume of 10 µL containing 1 µL of template DNA (at approximately 30–40 ng/µL), 1 µL of 10X buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris–HCl), 1 µL of dNTP mix (2 mM each), 1 µL of each primer (10 µM each), 0.1 µL of *Taq* polymerase (5 U/µL), and 4.9 µL of water. The thermal profile was: 95 °C for 1 min, 40 cycles at 95 °C for 30 s, 63 °C (COI) or 54 °C (16S) for 30 s, and 72 °C for 40 s, and final extension at 72 °C for 15 min. PCR products were visualized in 1% agarose gels and were purified with polyethylene glycol (8000 20%, NaCl 2.5 M) or with exonuclease I and shrimp alkaline phosphatase. Sequencing reactions were performed in both directions using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems), with the same primers used for PCR. Products were then precipitated using 75% isopropanol and loaded onto an ABI 377 sequencer (Applied Biosystems , Foster City, CA).

A consensus sequence per individual was obtained using Sequence Navigator (Applied Biosystems) or CodonCode Aligner 1.4.1 (CodonCode Corporation, Dedham, MA). Alignment, manual editing, revision of all the consensus sequences, and verification of ambiguities and unexpected stop codons were performed in BioEdit 7.0.9.0 (Hall 1999). In fulfillment of data archiving guidelines, we have deposited the primary data underlying these analyses in GenBank (Supplementary Table S1 online).

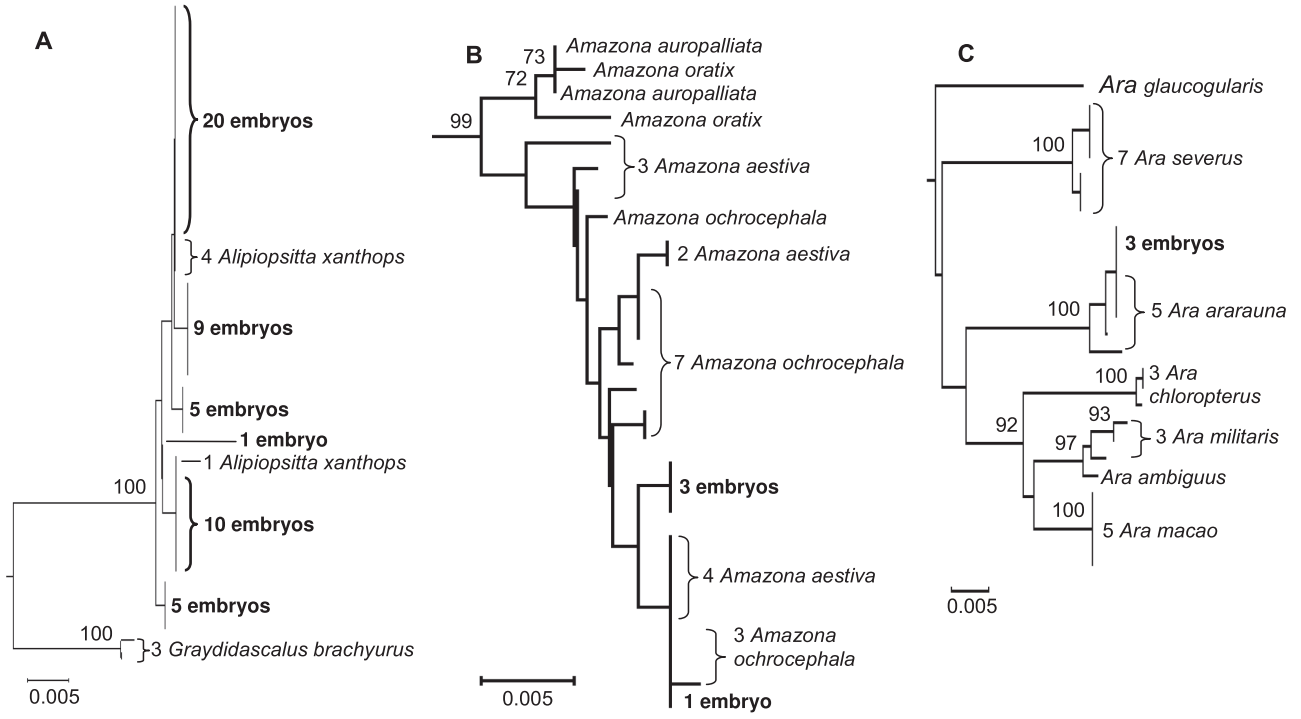

Figure 1. Segments of the neighbor-joining tree based on 564 bp of COI sequence, comprising 141 recognized species of parrots and 57 embryo samples of unknown identity. Bootstrap support values >70 are above the branches. (**A**) Fifty embryo sequences clustered with *Alipiopsitta xanthops*. (**B**) Four embryo sequences clustered with the [*Amazona aestiva*/*Amazona ochrocephala*] species complex. (**C**) Three embryo sequences clustered with *Ara ararauna*.

Each embryo's COI consensus FASTA sequence was used in the identification system in BOLD using the Kimura 2-parameter model (K2P; Kimura 1980). As the species of 57 of the embryos were identified as parrots (see Results section), their sequences were aligned with 351 sequences of 141 parrot species downloaded from BOLD. This data matrix was used to construct a neighbor-joining tree using the K2P model with 1000 bootstrap replicates (Felsenstein 1985) in MEGA 5 (Tamura et al. 2011). We also calculated the average K2P distance between each embryo and the closest parrot species identified. It is worth noting that BOLD does not yet include COI sequences from all bird species, but currently has sequences from 66% of all species in the family Psittacidae, and 83% of all parrot species that occur in Brazil. Police reported that the suspect did not leave Brazil during his visit; thus, if the eggs were from Brazil, BOLD held a high proportion of the possible matching sequences.

BLAST searches were conducted in GenBank using each of the 16S rDNA sequences to identify the species to which they were most similar. As the species of 57 of the embryos were identified as parrots (see Results section), their sequences were then aligned with 190 sequences of species from family Psittacidae downloaded from GenBank. A neighbor-joining tree based on this matrix and using the K2P model with 1000 bootstrap replicates was obtained in MEGA 5.

Results

COI Analyses

COI sequence lengths varied from 564 to 862 bp (Supplementary Table S1 online). The alignment matrix of the 58 embryo sequences contained 564 characters. The base composition of adenine (A), thymine (T), cytosine (C), and guanine (G) was 27.5%, 23.6%, 32.5%, and 16.4%, respectively. These frequencies were similar to those found in mitochondrial genes of other bird groups and in the COI of other animals (Tavares et al. 2006; Ward and Holmes 2007). We observed 12 haplotypes (Supplementary Table S1 online). The analyses in BOLD identified (similarity greater than 99%, Supplementary Table S1 online) 4 embryo samples as belonging to the parrot species complex [*Amazona aestiva*/*A. ochrocephala*] (second best hit: *Amazona oratrix*, *A. auropalliata*, or *A. barbadensis*; similarity range: 98.13–98.56), 3 samples as blue-and-yellow macaw *Ara ararauna* (second best hit: *Ara ambiguus* or *Ara militaris*; similarity range: 95.14–95.26), 50 samples as yellow-faced-amazon *Alipiopsitta xanthops* (second best hit: *Graydidascalus brachyurus* or *Pionus tumultuosus*; similarity range: 93.58–94.39), and 1 sample as an owl, *Megascops choliba* (second best hit: *Megascops clarkia*; similarity: 91.05).

Two subsequent analyses were conducted based on a parrot sequence matrix, so the sample identified as an owl was not included. In the neighbor-joining tree (Figure 1), embryo samples clustered with sequences of identified specimens with high bootstrap support values (99%), with the exception of the samples that clustered with the [*Amazona aestiva*/*A. ochrocephala*] species complex (bootstrap of 67%). These results agreed with those obtained with the identification analysis in BOLD (Supplementary Table S1 online). The K2P distances between the 57 embryo sequences and their corresponding closest identified parrot species ranged from 0.1% to 0.7% (Supplementary Table S1 online).

16S Analyses

Sequences ranged from 289 to 580 bp (GenBank accession numbers in Supplementary Table S1 online). The alignment matrix of the 58 embryo sequences contained 202 characters. When the sample with the shortest length was excluded (LGEMA 10872, 289 bp), the alignment matrix contained 332 characters. The base composition of A, T, C, and G for both matrices was 34.2%, 16.8%, 31.9%, and 17.1%, respectively. These frequencies are similar to those found in mitochondrial genes of other bird groups and the 16S rDNA of other

animals (Pereira et al. 2002; Tavares et al. 2006). Six haplotypes were identified (Supplementary Table S1 online).

Individual BLAST searches resulted in matches with ≥99% sequence identity (Supplementary Table S1 online) for all samples. As expected, the longer the sequence the smaller its e-value for the best hit. Sequences from group I (4 samples) were identified as belonging to the [*Amazona aestiva*/*A. ochrocephala*] species complex (second best hit: *Amazona oratrix*; e-value: 0.0 and identity range: 93–94%), those from group II (3 samples) as *Ara ararauna* (second best hit: *Ara glaucogularis*; e-value: 0.0 and identity: 96%), and those from groups III to V (50 samples) as *Alipiopsitta xanthops* (second best hit: *Graydidascalus brachyurus*; e-value range: 7.00E-176–0.0 and identity range: 90–92%). The single sequence in group VI was identified as an owl, *Bubo virginianus* (second best hit: *Bubo bubo*; e-value: 3,00E-134 and identity: 86%). The alignment of this sequence with parrot sequences revealed that they were highly divergent. Insertions and deletions were observed only in this sequence and only few identical bases occurred at the 5′ end. This sample was identified in BOLD as another owl species. Thus, 16S and COI analyses produced the same species IDs for all but 1 of the samples.

In the neighbor-joining tree (Supplementary Figure S1 online), 50 embryo sequences clustered with *Alipiopsitta xanthops* with maximum bootstrap support value (100%), confirming the BLAST results. The remaining 7 embryo samples clustered with sequences of species identified by BLAST, but with low bootstrap support values.

Discussion

Despite the shorter length of the 16S rDNA sequences, the analyses using both COI and 16S resulted in the same species identity for each of the 57 parrot embryo samples. Four samples were from the [*Amazona aestiva*/*A. ochrocephala*] species complex, taxa whose molecular diagnosis remains difficult (Eberhard and Bermingham 2004; Russello and Amato 2004; Ribas et al. 2007; Caparroz et al. 2009). Three samples were *Ara ararauna* and 50 were *Alipiopsitta xanthops*. The genera to which these parrot species belong are relatively well represented in BOLD (26 of the 66 species comprising the genus *Amazona* 8 of the 9 species of the genus *Ara*) and in GenBank (31 species of *Amazona*, 8 species of *Ara*). *Alipiopsitta* is a monospecific taxon and its sister species *Graydidascalus brachyurus* (Russello and Amato 2004) is also represented in BOLD and GenBank. This suggests that, even though these databases do not have sequences from all described avian species, the 57 parrot samples analyzed here were correctly identified.

The identification of 1 embryo sample was not clear. This sample was identified as *Megascops choliba* by BOLD analyses and as *B. virginianus* in GenBank analyses. Sequences from both owl species are deposited in BOLD, wheras in GenBank, only the 16S rDNA sequence of *B. virginianus* is available. Because BOLD holds sequences of both species, it seems more likely that this sample corresponds to *M. choliba*.

A valid criticism regarding the DNA barcoding approach to species identification is that it may not be able to distinguish closely related species (Moritz and Cicero 2004). However, relatively well-supported molecular phylogenies of parrot species suggest that the sister species of *Ara ararauna* is *Ara glaucogularis* (Oliveira-Marques 2006), the sister species of *Alipiopsitta xanthops* is *Graydidascalus brachyurus* (Russello and Amato 2004), and the most closely related taxa to the [*Amazona aestiva*/*A. ochrocephala*] species complex are *A. aurasiaca*, *A. versicolor*, and *A. barbadensis* (Eberhard and Bermingham 2004; Russello and Amato 2004). All these sister species are represented in both BOLD and GenBank, enhancing discrimination power. Thus, in this case we were able to identify the embryo species unambiguously, and our study comprises another example of the successful use of DNA barcoding in wildlife forensics (e.g. Teletchea et al. 2005; Dawnay et al 2007, Eaton et al. 2010, Asis et al. 2014).

We emphasize that the effectiveness of the identification method we used (comparison of the sequence of an unidentified sample with a sequence database) depends on having a comprehensive database with all taxa represented, as this greatly decreases the chance of species misidentification (Frézal and Leblois 2008, Coghlan et al. 2012). As BOLD is focused only on COI sequences, while GenBank holds sequences from various markers, currently BOLD seems to be more complete. Therefore, currently COI seems to be the best choice of molecular marker for species identification in most vertebrate groups.

Poaching is one of the major threats to species worldwide. Parrots are especially susceptible to this illegal activity; they are one of the most exploited groups among avian orders (Collar et al. 1992, Collar 1997). The employment of molecular techniques such as DNA barcoding has the power to identify taxa when morphological identification is not possible. Knowing the species targeted by poachers can help authorities develop more efficient protection plans for these species. In our case, most embryos were *Alipiopsitta xanthops* (50 of 58); if this pattern is repeatedly found, it could indicate that some poachers are very specialized. Regrettably, population-level markers are not available for all these species yet, making it impossible to infer from which population these animals may have been taken. The man who was carrying the eggs was fined with the minimum value per apprehended bird and released a few days after his arrest, and the information we produced was not used in the investigation. Unfortunately and in general, such cases are not treated as rigorously as, for example, an illegal drug apprehension.

In the future, a single nucleotide polymorphism database of the Brazilian (or even better, Neotropical) avifauna will be a necessary and powerful tool to identify species, populations, and individuals (Alacs et al. 2010). Much work is still needed in order to construct a solid foundation for incorporating the use of molecular tools in practical conservation of the Brazilian avifauna.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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