Brazilian Journal of Forensic Sciences, Medical Law and Bioethics

Journal homepage: www.ipebj.com.br/forensicjournal

The Use of DNA Barcoding to Identify Feathers from Illegally Traded Birds

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Received 20 December 2013

Abstract. The illegal trade of animals threatens the survival of wild populations, including birds, one of the most targeted groups. Brazilian native birds are protected by the current legislation, and their illegal trade is a crime that can be punished with imprisonment. However, in some cases, it is not easy to identify seized specimens, making the characterization of the crime difficult and so the punishment of the offenders. When morphological identification is compromised, genetic identification can be used to associate unknown samples to a reference sample by comparing sequences of mitochondrial genes. In this study I used a 650 bp region from the subunit I of cytochrome c oxidase gene (COI) associated with the Barcode of Life Database (BOLD) to identify five feathers seized by the Brazilian Federal Police in 2012. The DNA Laboratory of the Brazilian Federal Police was able to match four of the five feathers to the species *Triclaria malachitacea*, the Blue-bellied Parrot, a member of the family Psittacidae. The results support the idea that members of this group are preferred targets for the illegal trade of wild birds and proved that DNA barcoding is a valuable tool for forensic casework.

Keywords: DNA barcoding; Identification; Birds; Feathers; Psittacidae.

1. Introduction

The illegal trade of animals threatens the survival of wild populations, since the overexploitation to supply this activity can affect the ecosystems and cause extinctions[1]. This kind of illegal activity is widespread in many parts of the world, including Brazil[2], where is stimulated by the belief of impunity and the high profit rates

http://dx.doi.org/10.17063/bjfs2(4)y2013327

achieved. Estimates suggest that in Brazil the wildlife trafficking is responsible for the capture of 38 million specimens from the wild, a market estimated to worth about a billion dollars annually. According to the same report, birds represent 82% of the animals illegally traded in Brazil[3].

Brazilian native birds are protected by the current legislation, and their illegal trade is a crime that can be punished with imprisonment. However, in some cases, it is not easy to morphologically identify seized specimens, making the characterization of the crime difficult and so the punishment of the offenders. Contrary to what usually happens to adult birds, morphological identification of eggs, immatures and parts, such as feathers, is not always possible. When the morphological identification is compromised, genetic identification can be used in order to associate unknown samples to a reference sample by comparing sequences of mitochondrial genes that differ between species[4].

One of the most commonly used mitochondrial genes for species identification is the subunit I of cytochrome c oxidase (COI). Based on a region of 650 base pairs of this gene, a universal system for cataloging and identifying animal species, named DNA Barcoding, has been proposed[5,6]. A previous study has shown that the mean divergence between COI sequences of congeneric species of Chordata is 9.6%[7]. Additionally, a study with 260 North American bird species showed that all of them had different COI sequences and the difference between closely related species was 18 times higher than the differences within species[8]. Sequences of specimens with known identity that accomplish some quality criteria can be uploaded in the Barcode of Life Database (BOLD), an international publicly available reference database which can be used for species identification[9].

In Brazil, the genetic identification of birds in forensic casework is very restricted and carried only by a few institutions. This paper shows how the technique of DNA Barcoding was used by the DNA Laboratory of the Brazilian Federal Police to identify feathers seized in 2012, a case that probably would remain unsolved without the use of this genetic tool.

2. Material and methods

In 2012, during an operation to fight the illegal trade of wild animals, the Brazilian Federal Police seized five small (1.5 – 3.0 cm) greenish feathers inside a birdcage suspected to be used to contain protected species. The cage was found in the home of a suspect who, apparently, freed the bird(s) contained in the cage before the

arrival of the police. After initial assessment, the five feathers were sent to the DNA Laboratory for analysis.

After overnight digestion in extraction buffer with DTT and Proteinase K, DNA was extracted from individual feathers using standard phenol-chloroform procedures and purified with Amicon® Ultra (Millipore), following an adapted protocol previously described[10]. Fragments of approximately 650 bp from the 5' region of the COI gene were amplified using FishF1 and FishR1 primers[11]. The PCR were performed in 25 µl reaction tubes containing 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.4 mM of each primer and 1 µl DNA (DNA not quantified). The cycling parameters employed were 11 min at 94° C, followed by 35 cycles of 94° C for 30 s, 54° C for 30 seconds and 72° C for 1 min. Amplification products were purified using Exo-SAP-IT® (USB) and sequenced in both directions using Big Dye Terminator kit v1.1 (Life Technologies). The extension products were again treated with the enzyme alkaline phosphatase and purified by ethanol precipitation. Capillary electrophoresis was performed in an ABI 3130 genetic analyzer (Life Technologies).

Sequences were assembled and had their quality assessed with SeqScape v2.6 (Life Technologies) software. Consensus sequences were searched in BOLD Species Level Barcode Records database using the identification engine (www.boldsystems.org). BOLD identifies an unknown specimen to species level when there is less than 1% sequence divergence between the query sequence and the reference sequence.

3. Results

DNA extraction was successful for the five feathers and good quality identical 650 bp sequences were obtained from four of them. Analysis of the nucleotide sequences showed no signs of heteroplasmy and its translation into amino acids sequences did not reveal the presence of putative stop codons or pseudogenes. Due to the fact that the taxonomic identification of the seized feathers is uncertain, the obtained sequence was not uploaded to GenBank. The sequence obtained here can be requested directly from the author.

BOLD provided a species level identification for the query sequence. The best matched species was *Triclaria malachitacea*, also known as the Blue-bellied Parrot, a single species of the genus that belongs to the family Psittacidae. The species was represented by four different individuals in the database and the values of similarity between them and the query sequence ranged from 99.04 to 100%. The next five

other best matched species presented by BOLD are represented in the database by a variable number of individuals and are also members of the family Psittacidae. Similarity values between the query sequence and sequences of these other species were very low when compared to the best matched species (Table 01).

Table 1. Results of BOLD search presenting the six best matched species and their best similarity values.

Order	Species name	Best similarity value per species (%)
1st	*Triclaria malchitacea*	100
2nd	*Amazona albifrons*	91.67
3rd	*Amazona ochrocephala*	91.39
4th	*Pionus chalcopterus*	91.36
5th	*Amazona guildingii*	91.20
6th	*Graydidasculus brachyurus*	91.05

4. Discussion

The effectiveness of BOLD for species identification depends basically on two factors. First, the COI sequence divergence must allow the differentiation between species, even the closely related ones and, second, the reference database must represent the diversity of the group. A study with 561 species of Neotropical bird species showed that 93% of them have unique COI sequences and can be correctly identified based on their barcodes, similarly to the pattern found in other regions of the world[12]. Besides, another study pointed that, although some species can present much lower values, the mean interspecific COI sequence divergence found for Neotropical Psitaccidae was approximately 12%[13]. Additionally, according to phylogenetic studies based in mitochondrial and nuclear sequences, the single species genus *Triclaria* is more related and forms a clade with the genera *Amazona*, *Pionus*, *Graydidasculus*, *Pionopsitta*, *Myopsitta* and *Brotogeris* of Neotropical parrots[14]. In July 2013 BOLD contained in its database barcodes of all the species of these genera that occur in Brazil15, which is very representative. I consider that, at least in the present case, BOLD produced robust results and the identification of *T. malachiatacea* as the species of the origin of the seized feathers is conclusive.

Due to the color of its feathers, behavior and vocal capabilities, members of the family Psittacidae are quite popular among collectors, and the price of each

specimen can reach thousands of dollars on the international market[1,16]. This is one of the reasons why the trade of most species of the group is regulated by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). This Convention, of which Brazil is a signatory, aims to prevent that the trade of species of wild fauna and flora threatens their survival in nature. Although in Brazil there are not many studies on the subject, data related to a wildlife reception center of the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA), pointed the family Psittacidae as second in number of animal species and the fourth in number of individuals seized or voluntarily delivered to that facility[17]. The results obtained here, as well as other seizures made by the Brazilian Federal Police in previous years[18], support the idea that the members of this group are preferred targets for the illegal trade of wild birds.

The fight against illegal trade in wild species, whether for domestic or international trade, is essential for the protection of the Brazilian avifauna. Among the most threatened species are several members of the family Psittacidae, charismatic birds with high demand on the black market. Although protected by the Brazilian laws, T. malachitacea is not threatened of extinction like other members of the group. However, techniques like DNA Barcoding and other genetic tools must be improved and widely used in forensic casework, helping to prevent the decline of natural populations of this species and avoiding the extinction of many others.

Acknowledgements

The author would like to thank Jorge Freitas and Gustavo Chemale for their comments and suggestions in the improvement of this manuscript.

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