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European Roller thief nailed by DNA found in a bird carrier

Luisa Garofalo[1]*, Rita Fanelli[2], Annalisa Brucoli[3], Sebastian Cannarella[4], Rita Lorenzini[5]

[1]Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M. Aleandri" - Via Appia Nuova 1411, 00178 Roma, Italy

[2]Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M. Aleandri" - Strada Terme, 01100 Viterbo, Italy

[3]Raggruppamento Carabinieri CITES, Reparto Operativo, Sezione Operativa Antibracconaggio e Reati a Danno degli Animali (SOARDA) - Via Carducci 5/E, 00187 Roma, Italy

[4]Associazione Ornis Italica - Piazza Crati 15, 00199 Roma, Italy

[5]Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M. Aleandri", Centro di Referenza Nazionale per la Medicina Forense Veterinaria - Viale Europa 30, 58100 Grosseto, Italy

*corresponding author: luisa.garofalo@izslt.it

LG 0000-0002-3652-272X

Abstract - In 2018 a bird poacher, who used to remove chicks illegally from their wild nests in Central Italy, was the focus of a complex investigation by law-enforcement. Several clues were found suggesting his illegal activity against wildlife, and some items were seized by the authorities from his home, including a carrier probably used to restrain birds. The bird carrier was delivered to the Forensic Genetics Laboratory (Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M. Aleandri") where evidence of the offence was looked for through molecular tools. To identify the species of origin for the poached birds, portions of two mitochondrial markers (Cytochrome b and Cytochrome Oxidase I) were amplified and sequenced using the DNA isolated from biological traces that were found inside the carrier. Sequences from the evidence samples were compared for homology with those lodged in online genetic repositories. The results revealed that at least one individual of European roller *Coracias garrulus*, a protected bird species listed as vulnerable in the Italian Red List, had been restrained in that pet carrier. Species identification was further confirmed through comparison with sequences from an in-house *C. garrulus* sample. Eventually, the suspect and seven more people were formally charged with poaching of protected species, and a long list of further crimes.

Keywords: animal forensic genetics, *Coracias garrulus*, illegal bird trade, molecular markers, species identification

INTRODUCTION

European Rollers *Coracias garrulus* (Linnaeus, 1758) are small, colourful long-distance migrants. Two subspecies are currently recognized (BirdLife International 2019): the nominate *C. g. garrulus,* which breeds from North-Western Africa, South-Western, South-Central and Eastern Europe and Asia Minor eastward through North-Western Iran to South-Western Siberia (Russia), and the Asian subspecies, *C. g. semenowi*, which breeds in Iraq, Iran (except North-Western), Pakistan, Kashmir, South Kazakhstan, Turkmenistan, and North-West China (del Hoyo et al. 2001, Tokody et al. 2017). Recently,

phylogeographic analyses based on sequencing of the mitochondrial Control Region confirmed the split of the species into two haplogroups: one comprising European haplotypes and one including Asian haplotypes (Nebel et al. 2018).

The European Roller breeds throughout temperate, steppe and Mediterranean zones characterised by a warm summer weather, preferring lowland open countryside, oak and pine forests with clearings, wintering primarily in dry wooded savanna and bushy plains (del Hoyo et al. 2001). Different breeding populations use different but overlapping wintering areas, and individuals from the same population can use different wintering sites (Rodriguez-Ruiz et al. 2014, Finch et al. 2017). Rollers that breed in European countries usually overwinter on the African continent. Ringed individuals departing from Southern France were recovered, after migration, in South-Western Africa (Emmenegger et al. 2014), whereas Central and Eastern European rollers were found in wintering sites across Sub-Saharan Africa (Finch et al. 2017). Satellite transmitters tracked South-Western Iberian individuals moving towards the South-African Atlantic coast (Rodriguez-Ruiz et al. 2014).

Rollers feed mainly on arthropods and sometimes on small vertebrates (e.g. lizards, amphibians) in agricultural habitats, nesting in natural or artificial holes (Catry et al. 2019). Loss of suitable breeding habitat due to changes in agricultural practices, reduction of nesting sites and use of pesticides (that reduce food availability) are considered to be the main threats to the species in Europe (Kovacs et al. 2008, Tokody et al. 2017). Further threats include persecution and shooting during migration in some Mediterranean and Arabian countries (del Hoyo et al. 2001, Finch et al. 2017). A recent survey on illegal hunting in the Mediterranean revealed that *C. garrulus* is one of the twenty species of major conservation concern showing the highest number of birds killed (relative to population size), particularly in Syria, Cyprus, Egypt and Lebanon (Brochet et al. 2016). The species is globally assessed as Least Concern in the IUCN Red List of Threatened Species (BirdLife International 2019). Although the Central Asian population is apparently not experiencing a significant reduction, the European population trend is decreasing. Following a moderate drop during 1970-1990, the species has continued to decline up to 25% during 1990-2000 across the whole Europe (BirdLife International 2004). In some Northern and Central European countries it went extinct. Overall, a 5-20% decline was recently estimated in Europe over the past three generations (16.8 years, BirdLife International 2015). First signs of genetic drift were reported for the European Roller from Austria, where a drastic decrease in population numbers also caused a reduction in mitochondrial diversity (Nebel et al. 2018).

Italy is the Southern European country that acts as a bridge towards Africa. Here, the species is included in the Italian Red List as Vulnerable (VU) according to the D1 criteria, due to small population size and distribution area. It is listed in the Annex I of the Birds Directive (79/409/EEC, now replaced by the Council Directive 2009/147/EC on the conservation of wild birds) and is protected according to Article 2 of the Italian Law 157/92. The Italian population is estimated to be 300-500 breeding pairs (Brichetti & Fracasso 2007), and although updated numbers have reached 1,000 pairs in 2011-2013 (Meschini 2015), the current population size is unknown. Rollers are mainly distributed in Central-Southern Italy, with nests occurring in Tuscany, Latium, along the coasts of Apulia, Calabria, Sicily and Sardinia (Brichetti & Fracasso 2007). In Italy, and elsewhere in Europe, the species is frequently subject to poaching and illegal trade in live animals, mainly due to their bright attractive colours (BirdLife International 2019).

Wildlife forensic genetics has recently emerged as a powerful tool to tackle crimes against wild birds (e.g. Abe et al. 2012, Coghlan et al. 2012, White et al. 2012, Presti et al. 2015), and is becoming increasingly important in caseworks that come to trial. In this short-note we report on a casework of illegal removal of live roller(s) from the wild in Italy. The offence

was ascertained through molecular identification of the species by sequencing of highly informative mitochondrial markers.

MATERIALS AND METHODS

Case history and samples

In 2018 a suspected bird thief, who used to illegally remove chicks of different bird species from their wild nests, was the focus of a complex investigation by law-enforcement (i.e. Italian Police Department of Carabinieri CITES). An ornithological association, Ornis Italica, reported on the theft of nestlings from nest-boxes located in the northern area of Latium region as part of a project aimed at monitoring the Central Italian population of European Rollers and at improving nest site availability for the species (Monti et al. 2019).

Several clues were found suggesting the illegal activity of the suspect against wildlife, and some items were seized by the authorities from his home, including a carrier probably used to restrain birds. The bird carrier was delivered to the Forensic Genetics Laboratory of Centro di Referenza Nazionale per la Medicina Forense Veterinaria (Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M. Aleandri", IZSLT) where evidence of the offence was looked for through molecular tools.

The carrier seized from the suspect was visually inspected in the laboratory and several items were found inside, including dirty straw on the bottom, grains, biological traces on fabric and paper handkerchiefs. A total of seven samples were collected: feathers stuck on a black tape (N=3), blood traces on the inside of the lid (removed using a swab, N=1), stool on a denim fabric (N=1) and on paper handkerchiefs (N=2) (Fig.1).

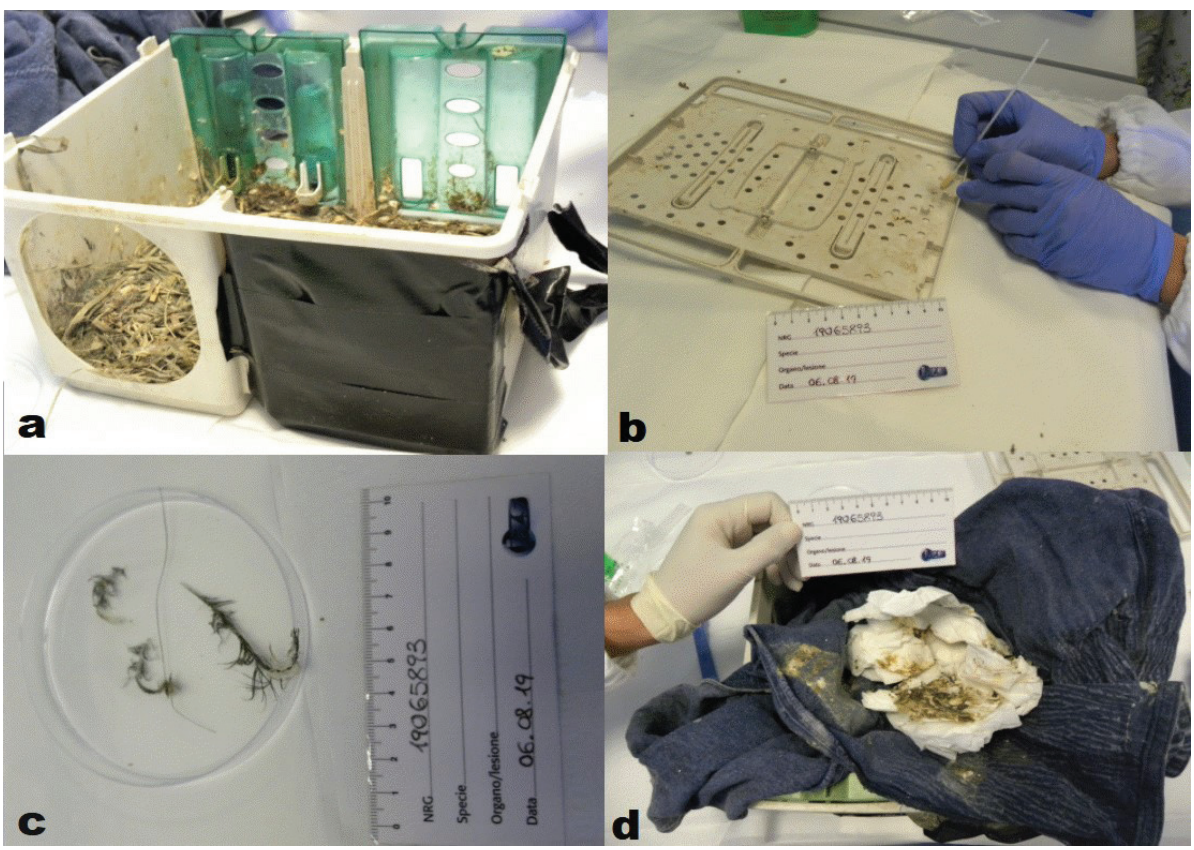

Figure 1. Pet carrier seized from a bird thief. (a) Exterior and interior of the carrier. Samples collected inside: (b) swabbing of the blood traces, (c) three feathers, (d) stool on the denim fabric and on paper handkerchiefs.

Laboratory analyses

DNA was isolated from the quill of the feathers and from the swab using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Minor adjustments during DNA extraction consisted of adding 20 µl 1M DTT (Dithiothreitol) to 180 µl ATL Lysis Buffer (Qiagen, Hilden, Germany) and 20 µl proteinase K (20 mg/ml) in the digestion step at 56°C overnight. DNA from stool was extracted according to the instruction of the QIAamp DNA stool MiniKit (Qiagen, Hilden, Germany) and eluted in 100 µl elution buffer provided. A mock tube with reagents and no DNA was included in each extraction session.

We selected two mitochondrial genes traditionally recommended for bird species identification: Cytochrome b (Cytb) and Cytochrome Oxidase I (COI) (Branicki et al. 2003, Dawnay et al. 2007). The gene fragments were amplified using both a newly designed primer and primers published previously (Tab.1): ND5-mod_FOR (this study) and H15149 (Kocher et al. 1989) for Cytb, ST_f and IN3r (Schäffer et al. 2017) for COI. In Schäffer et al. (2017) several primers were provided for COI sequencing of mammals and birds, but the combination of ST_f and IN3r had never been applied before. The software Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) was used for primer design.

PCR reactions contained 2.5 µl of 10X Gold buffer (Applied Biosystems), 200 µM of each dNTP, 2.5 mM $MgCl_2$, 10 pm of each primer and 1U of AmpliTaq Gold polymerase (Applied Biosystems), 3 µl template DNA, in a final volume of 25 µl. Amplifications were performed in a Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA), and consisted of an initial 5 min denaturation step at 95°C, 39 cycles of 30 s at 95°C, 30 s at the annealing temperature (Tab.1), 2 min extension step at 72°C, followed by 5 min at 72°C. PCR negative controls were included in each amplification round. Amplified products were visualised under UV light after electrophoresis on 1.5% agarose gel and staining with Gel Red™ (Biotium, Inc., Hayward, CA, USA). A 50–2,000 bp DNA ladder (Sigma-Aldrich Chemicals, Milan, Italy) was used to verify the molecular sizes of amplicons. PCR products were cleaned up with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced bidirectionally using the amplification primers and the BigDye Terminator kit v3.1 (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. Unincorporated dyes and other contaminants were removed with the Agencourt® CleanSEQ solution (Beckman Coulter, Beverly, MA, USA), then sequences were loaded onto a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and analysed using the Sequencing Analysis Software v5.3.1 (Applied Biosystems, Foster City, CA, USA). Sequences were edited and aligned using the multiple alignment program included in the package VECTOR NTI v. 9.1 (Invitrogen, Carlsbad, CA, USA). DNA from evidence samples was amplified and sequenced in triplicate. The sequences obtained were entered in the National Centre for Biotechnology Information Database (NCBI), and homology was evaluated to the sequences available in the GenBank database (www.ncbi.nlm.nih.gov) through the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi), that lists the similarity in descending order of percentage of identity.

Table 1. Details on mitochondrial primer pairs and amplification conditions. The size in base pair (bp) refers to the entire fragment, primers included.

mtDNA region	SIZE (bp)	PRIMER FORWARD (5'-3')	PRIMER REVERSE (5'-3')	ANNEALING TEMPERATURE
COI	489	**ST_f** CYNCWAMCCACAARGAYATNGGNAC	**IN3r** GATCANACGAANAGNGGNGTYTG	53°C
Cyt b	437	**ND5-MOD_FOR** TATCTAGGATCTTTCGCCCT	**H15149** AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA	55°C

RESULTS

Amplification of Cytb (437 bp in length, Tab.1) was successful for three stool samples (Fig. 1d). The remaining evidence samples collected from inside the carrier (feathers and blood traces) yielded no results. Once aligned, the sequences obtained from stool showed no site variation. Three replicates from each stool sample yielded concordant results. When submitted to the BLAST query, the sequence from stool samples returned a percentage of identity (PI) of 99.02% (3-bp difference) with the following accession number AF407448, a sequence belonging to *Coracias garrulus* from Pakistan: the only Cytb sequence of this species which is registered in GenBank at the time of writing this communication. PI of 92.91% (27-bp difference) and 92.39% (29-bp difference) were found with *Coracias caudatus* (U89184.1) and *Coracias spatulata* (AF082060), respectively.

A further check of the results obtained from stool was carried out using a known sample of European roller from the Italian population. The bird died accidentally years ago, and muscle tissue was stored in the biobank of the Forensic Genetics Laboratory at IZSLT as reference sample. DNA was extracted from approximately 25 mg of muscle with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and amplified with the same protocols described above, with a number of cycles in the PCR reaction decreased to 35. Extraction, amplification and sequencing of the DNA from this individual (High Copy Number DNA sample) were performed separately, after the analysis of the evidence (low template DNA samples). A Cytb sequence of the same length (437 bp) was obtained from the known *C. garrulus* sample and deposited in GenBank (www.ncbi.nlm.nih.gov) with accession number MW401760. This sequence differed from that of the stool samples by one base-pair mutation (C/G at position 209 of the 383-bp Cytb alignment).

Amplification of the COI fragment (489 bp in length, Tab.1) was not successful for all seven evidence samples. When PCR products were checked on the agarose gel, no bands of good quantity and quality were detected under the UV light. In order to verify the amplification conditions employed, COI was also amplified in the European roller sample used previously in the Cytb comparison. A successful amplification yielded a 441-bp-long sequence that was registered in GenBank with accession number MW402994.

DISCUSSION

In 2018, an ornithological association operating in Central Italy reported the theft of roller chicks from their nests. The Italian Police Department of Carabinieri CITES started an investigation which led to the seizure of a pet carrier at a suspect's home. Feathers, stool and blood traces were found inside the carrier and collected as evidence samples.

Of the two mitochondrial gene fragments used to identify the species of origin of the evidence samples, the longer one (COI, 489 bp) yielded no amplicons. This was likely due to the high degradation of DNA, which usually prevents medium-long size gene fragments from being amplified. By contrast, amplification of the shorter segment (Cytb, 437 bp) from the evidence samples was successful, yielding a product of the expected size. Comparisons of the obtained sequence with the online sequences from the GenBank public repository and with a known sample of European roller led to the identification and subsequent confirmation of the unknown DNA as belonging to the species *C. garrulus*.

Genetic results, in combination with additional clues collected during the investigations, ensured that the suspect was formally charged by the law-enforcement authorities with a long list of crimes: i) theft to the detriment of the Italian State (Arts. 624 and 625 of the Penal Code); ii) animal abuse (Art. 544-ter); iii) trade in stolen goods (Art. 648); iv) counterfeiting of State seals (Art. 468); v) unlawful use of State seals (Art. 471), because avian ID rings, considered equivalent to State seals by the Italian laws, were fraudulently used to fake the illegal origin of the animals; vi). commercial fraud (Art. 515); vii) collusion (Art. 110); viii) repeat offences (Art. 99), because he had already been convicted of similar

crimes; ix) reiteration of a continuous crime, seen as a series of acts to pursue the same criminal design (Art. 81); x) violation of Art. 30, c.1, lett. b of the Italian Law 157/92, which regulates the management, conservation and hunting of wildlife. Eventually, the investigations revealed that the man was managing an unprecedented national, but also international, illegal trade of birds that were systematically removed from the wild using nets, traps, by directly stealing chicks from their nests, and other ways. Seven more people were also investigated for those crimes.

Molecular markers have repeatedly demonstrated their usefulness in animal species identification when applied to wildlife crimes, such as poaching (e.g., Iyengar 2014, Lorenzini & Garofalo 2021). The present casework confirms the increasing importance of wildlife forensic genetics in supporting law-enforcement investigations to protect endangered and threatened bird species in areas that are crucial for their conservation during the breeding and/or migration periods. This holds particularly true for the Italian and French populations of *C. garrulus*, that have recently been identified as key populations for the conservation of the species in Europe, in the face of climate change (Kiss et al. 2020). Furthermore, forensic genetics can be a valid conservation tool for many other Afro-Palaearctic landbirds that choose to migrate across Italy (Briedis et al. 2019), where they often fall prey to poachers.

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