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Short Communication

DNA barcoding against poaching of Chamois (*Rupicapra rupicapra*), two confirmed cases from Greece

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Abstract

Poaching has been reported as a major threat to the survival of many species including the Chamois (*Rupicapra rupicapra*). We report two recent cases from Greece where COI and Cytb sequences were used for species identification from evidence samples. Not only we confirm the poaching events, but through the description of the laboratory procedures we highlight two limitations of the barcoding method, COI-like sequences and the universality of COI primers.

Keywords: Wildlife forensics, COI-like sequences, Cyt*b*, illegal hunting.

Introduction

The illegal hunting of animals, hereafter poaching, can seriously affect the dynamics of populations, threatening the viability of many species worldwide (Gavin *et al*. 2010). In addition, a less obvious but equally severe effect is the potential cause of genetic changes that can hamper management efforts (Allendorf *et al*. 2008). In poaching cases, a common problem for law enforcement is the identification of the illegally killed species from evidence such as blood and tissue, when other morphological features are absent. When animal hair is included as evidence, a great deal of expertise is required to accurately identify the species in question with a high degree of confidence. Even so, microscopic examination of hairs may not yield a definitive identification (Linacre and Tobe 2011). This is where the ability of DNA techniques to accurately identify a sample to the species level, come into focus. The amplification and sequencing of mitochondrial fragments such as the cytochrome *b* gene (Cyt*b*) has been used in various wildlife forensic cases (Verma and Singh 2003) but the cytochrome c oxidase subunit I (COI), has become the gene of choice for the molecular species identification. A 650bp fragment of COI can be used to query large COI databases (e.g. BOLD) and determine the identity of unknown samples. This method is known as "DNA barcoding" (Hebert *et al*. 2003).

The Balkan Chamois (*Rupicapra rupicapra* balcanica) is a mountain ungulate species of the subfamily caprinae that spreads throughout the Balkan Peninsula, from Croatia and Serbia to central Greece, which is the southern limit of its distribution. Overall, its populations are declining (Corlatti *et al.* 2011), whereas in Greece the population is estimated at 480-750 individuals, showing a fragmented distribution pattern (Papaioannou and Kati 2007).The species is under strict protection in the country thus its hunting has been prohibited since 1969. However, poaching has been identified as a major threat for the species' survival while various incidents regarding the confirmation of illegally hunted animals in the field, are increasing at an alarming rate (Papaioannou *et al.* 2015). Here, we report two recent cases of

Chamois poaching in Greece, and describe the steps taken for the molecular species identification from evidence samples. We aim to highlight this major threat that can severely halt the growth of populations in the region along with assessing the barcoding method in respect to the different biological material used and the limitations that were encountered.

In the morning of February 21, 2017 the Game Guard Body of Ioannina (NW Greece) was on a routine patrol when they heard gunshots in the area of Tsepelovo Village. The wardens set a roadblock and pulled over a car with three male passengers. No guns or hunted animals were detected, passengers claimed that they were searching for their lost dogs and were eventually released. However, after searching the area, the wardens spotted six recently deceased Chamois that were hidden under some branches indicating that the assailants had probably already detected the presence of the Game Guard in the area. After a second subsequent roadblock the same party was searched thoroughly and traces of fresh blood and hair were found in the car. The three persons were arrested while a fourth man that allegedly fled the scene carrying the weapons was declared wanted by the police. After examination by a veterinarian, the six Chamois showed clear firearm injuries. Consequently, the identification of the species from the evidence in the seized car was requested. Blood samples were collected using blood storage cards (NucleoCards®), hairs were stored in paper envelopes while fresh tissue samples taken from the dead animals to be used as reference were stored in 99.8% ethanol until DNA extraction. The case quickly gained publicity after numerous press releases (http://www.epirus–tvws.gr /2017 / 02 / 6_22 html). The court case is currently ongoing.

In the second case, the Management Body of Tzoumerka, Peristeri and Arachthos Gorge National Park (NW Greece) received information concerning the poaching of Chamois that possibly happened on 15 September 2017. In-situ investigation by the wardens of the Management Body took place after five days when evidence of a killed animal were discovered, including dried blood stains, one small piece of dried tissue and numerous hairs. Chamois droppings were also detected nearby as well as footprints revealing human presence in the scene. Identification of the species on the basis of the above-mentioned biological material was also requested in order to lay charges.

Laboratory procedures

Genomic DNA was isolated from a total of 18 samples, 12 from the first case (four blood and two hair samples from evidence as well as six tissue samples, each belonging to the deceased Chamois) and six from the second case (two dried blood, two hair and two dried tissue samples). DNA was extracted using the NucleoSpin Tissue kit (Macherey-Nagel) following the manufacturer's protocol. We amplified a 650 bp fragment of the cytochrome oxidase subunit I (COI) gene using the universal primers HCO2198: 5'-TAAAC TTCAGGGTGACCAAAAAATCA-3' and LCO1490: 5'-GGTCAACAAATCATAAAGA TATTGG-3' (Folmer *et al*. 1994). Due to the suspected degradation, samples from the second case were additionally amplified in a smaller 307 bp fragment of the cytochrome b gene (Cyt*b*) using the vertebrate-specific universal primers L14841: 5'-AAAAA GCTTCCATCCAACATCTCAGCATGATG AAA-3' and H15149: 5'-AAACTGCAG CCCCTCAGAATGATATTTGTCCTCA-3' (Kocher *et al.* 1989). Polymerase chain reaction (PCR) amplification for both genes was carried out in a total volume of 20 mL using the KAPA taq PCR Kit (Kapa Biosystems). The final concentration of the reagents were as follows: 1x PCR buffer, 2.5 mM MgCl2, 200 μM of each dNTP, 0.5 μM of each of the forward and

reverse primer, 1 unit (U) Taq DNA polymerase and 20-30 ng of genomic DNA template. PCR profile included an initial denaturation step of 3 min at 95 °C, 35 cycles of 30 s at 95 °C, 60 s at 60 °C (COI)/ 55 °C (Cytb), 90 s at 72 °C, with a final extension step of 10 min at 72 °C. PCR products were purified using the Nucleospin® Gel and PCR Clean-up kit and Sanger-sequenced in forward and reverse. Sequence trace files were examined by eye, aligned in MEGA 7 (Kumar *et al.* 2016) and compared against GenBank and BOLD to identify the species in question. MEGA 7 was also used to construct a Neighbour-Joining tree based on our dataset and additional sequences retrieved from GenBank for both COI and Cyt*b*. Taxonomically related species such as *R. pyrenaica* and Capra ibex were used as outgroups. We used the Kimura-2-parameter model of nucleotide substitution and the robustness of nodes was assessed by 10000 bootstrap replications. All sequences were deposited in GenBank.

Results and Discussion

All 18 samples from both cases yielded quantifiable DNA. For the first case the COI fragment was amplified successfully for all samples whereas for the second only two out of six samples (one hair and one blood sample) yielded PCR products. When samples from the second case were additionally amplified for the Cytb fragment, all six were successful. Finally, readable COI sequences were obtained from a total of seven samples (six from the first case and one hair sample from the second). Regarding Cytb, we sequenced five samples, but two were unsuccessful including one blood and one dried tissue sample. In general, hairs proved to be the sample type that performed better than others, providing the most readable sequences in this study (Table 1).

For the first case, we found two different haplotypes in the six COI sequences, which when queried against BOLD, were found to be from Chamois (*R. rupicapra*) with high degree of confidence (similarity 99.8% and 99.6% respectively). Both haplotypes were found in samples from the dead animals, further supporting the confident identification of the evidence. However, those two haplotypes were highly divergent which was reflected in the high p-distance (15.8%) between them. After further examination, one of the sequences showed the presence of stop codons suggesting that actually a "COI-like" sequence was amplified. Although COI-like sequences can provide valuable phylogenetic information (Pérez *et al.* 2017), they can lead to erroneous analyses if they are not reported as such when deposited in sequence databases (Buhay 2009). In our case, COI-like sequences were clustered with unpublished sequences of Chamois originating from Croatia (Fig. 1). This indicates that these deposited sequences are probably also COI-like, although not mentioned so in GenBank.

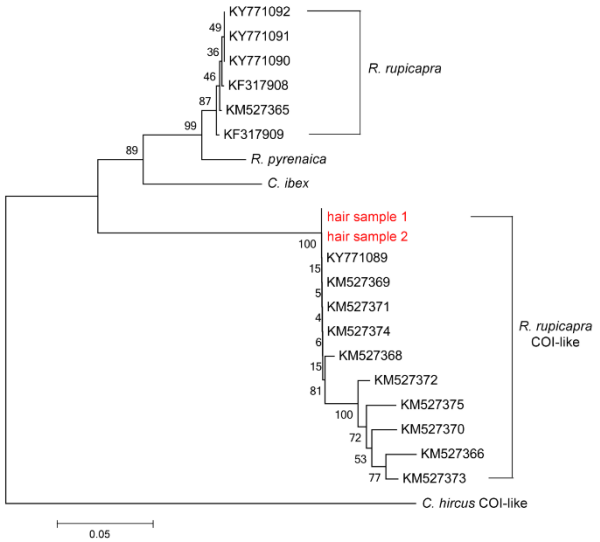

Figure 1. Neighbour-Joining tree showing phylogenetic relationships between samples obtained from evidence (in red; accession numbers KY771087, KY771088 respectively) and COI sequences retrieved from GenBank. We used the Kimura-2P model of nucleotide substitution. Numbers at nodes indicate bootstrap support and scale bar represents substitutions per nucleotide position.

Therefore, we urge researchers to carefully examine sequences before deposition in order to avoid misleading results regarding phylogeographic and wildlife forensic studies. For the second case, the COI sequence from the hair sample proved to originate from a tardigrade species (Echiniscus testudo). This highlights another limitation of the barcoding method since the universality of the primer set along with the contamination of our samples due to their prolonged exposure in the field, led to misleading identification. However, Cytb sequences showed that our unknown samples actually belonged to Chamois (Fig. 2). It is essential that, when possible, more than one marker should be evaluated in wildlife forensic cases that involve species identification to increase the robustness of the conclusions (Linacre and Tobe 2011). Due to the current population status of the Chamois in Greece and its slow recovery rates, poaching can have devastating effects on the population level. Although combating poaching can be very challenging, we urge authorities to take urgent measures against it such as the implementation of a more effective guarding system and road control. From then on, if a poaching event occurs, DNA barcoding can be used to confidently confirm such cases thus helping to build solid court cases.

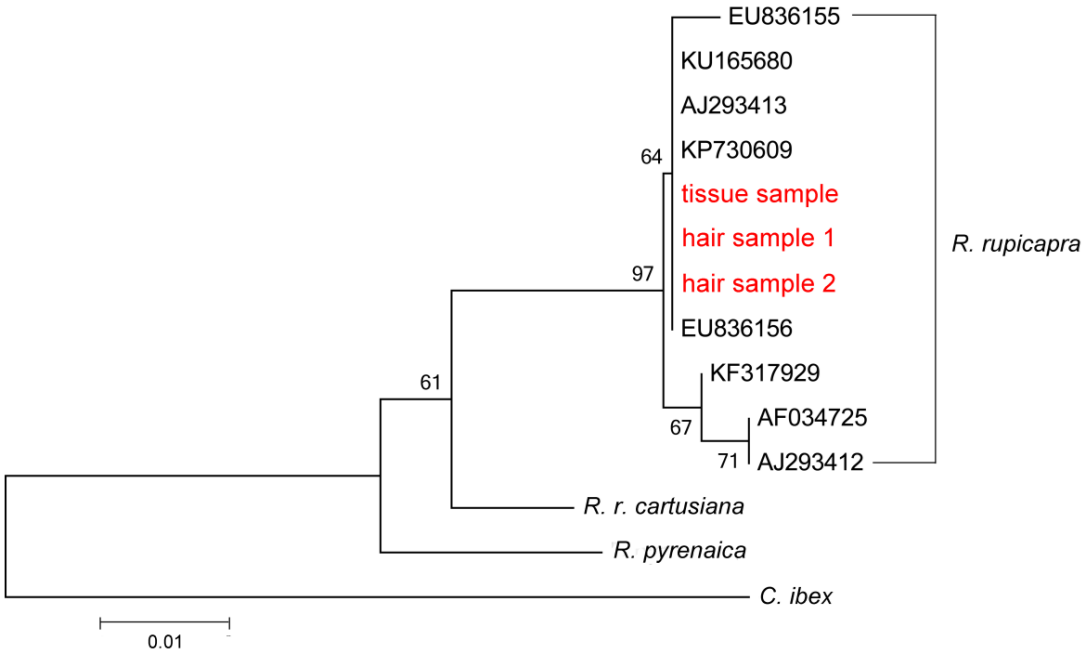

Figure 2. Neighbour-Joining tree showing phylogenetic relationships between samples obtained from evidence (in red; accession numbers of haplotype from both hair samples: MG457176; tissue: MG457177) and Cytb sequences retrieved from GenBank (accession numbers shown). We used the Kimura-2P model of nucleotide substitution. Numbers at nodes indicate bootstrap support and scale bar represents substitutions per nucleotide position.

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